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BIODEGRADATION OF  
NITROGLYCERIN AS A GROWTH  
SUBSTRATE:

A BASIS FOR NATURAL  
ATTENUATION AND  
BIOREMEDIATION

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# BIODEGRADATION OF NITROGLYCERIN AS A GROWTH SUBSTRATE:

## A BASIS FOR NATURAL ATTENUATION AND BIOREMEDIATION

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This dissertation is dedicated to my husband Joan, my mother, Myriam, and my brother James.

In memory of my father James V. Husserl

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## CONTENTS

Acknowledgements .....	iv
List of Tables .....	viii
List of Figures .....	ix
Project Summary .....	xi
Chapter 1: Introduction .....	1
Chapter 2: Literature Review .....	6
2.1 Nitroglycerin Contamination .....	7
2.2 Biological Degradation of Nitroglycerin .....	8
Chapter 3: Growth of <i>Arthrobacter</i> sp. strain JBH1 on Nitroglycerin as the Sole Source of Carbon and Nitrogen .....	16
Chapter 4: Enzymatic Reactions that Allow <i>Arthrobacter</i> sp. strain JBH1 to Grow on Nitroglycerin as the Sole Source of Carbon and Nitrogen .....	20
4.1 Abstract .....	20
4.2 Introduction .....	20
4.3 Materials and Methods .....	22
4.4 Results .....	27
4.4.1 Transformation of NG to 1 MNG .....	27
4.4.2 Transformation of MNG .....	29
4.5 Discussion .....	34
Chapter 5: Biodegradation of Nitroglycerin in Porous Media .....	39
5.1 Abstract .....	39
5.2 Introduction .....	39
5.3 Materials and Methods .....	41
5.4 Results and Discussion .....	45
5.4.1 Column Experiments .....	45
5.4.2 Shake Flask Experiments .....	52
Chapter 6: Conclusions and Engineering Significance .....	56
Appendix A: NG Transport and Definition of Natural Attenuation .....	60
A-1 Advection .....	61
A-2 Hydrodynamic Dispersion .....	62
A-3 Sorption of NG to Soil Media .....	63
Appendix B: Hydrolysis of Nitroglycerin in Aqueous Solution as a Function of pH .....	66
B-1 Materials and Analytical Methods .....	67
B-2 Results and Discussion .....	68
Appendix C: Soil Characterization and Sorption of NG to Soil Medium .....	71

C-1 Soil Characterization .....	71
C-2 Results and discussion.....	72
Appendix D: Mixed Culture Capable of Growing on NG as the Sole Source of Carbon and Nitrogen.....	74
D-1 Theoretical considerations.....	74
D-2 Methodology .....	76
D-3 Results and Discussion.....	77
Appendix E: pH and Substrate Inhibition.....	83
E-1 Methodology.....	84
E-2 Results and Discussion. ....	85
References .....	95

## LIST OF TABLES

---

Table 2.1. NG contamination at different sites	8
Table 2.2. Summary of recent publications that investigate the microbial degradation of NG	11
Table 4.1. Bacterial strains, plasmids, and primers used in this study	26
Table 4.2. Identities of the sequence alignments of OYEs identified in JBH1 and other OYEs known to denitrify NG	28
Table 4.3. Transformation of 1 MNG in JBH1 cell extracts under different experimental conditions	30
Table 5.1. Soil Characteristics	43
Table C.1. Fraction of organic carbon in soil	73



## LIST OF FIGURES

---

Figure 2.1. Nitration of glycerol to produce nitroglycerin	6
Figure 2.2. Nitroglycerin biological transformation pathway when used as a nitrogen source	9
Figure 4.1. NG transformation in cell extracts after addition of NADPH (left) and 1 MNG transformation in cell extracts after addition of Mg-ATP (right)	27
Figure 4.2. Amount of 1 MNG produced in <i>E. coli</i> clones containing overexpressed OYE1, OYE2, OYE3, OYE4, and the control	29
Figure 4.3. 1 MNG transformation rate in cell extracts from JBH1, <i>E. coli</i> cells with overexpressed glycerol kinase from JBH1, and <i>E. coli</i> control	32
Figure 4.4. Annotation of pJBH1A3 (region of interest)	33
Figure 4.5. Proposed transformation pathways of 1 MNG catalyzed by the glycerol kinase in JBH1	36
Figure 5.1. Transient change in NG effluent concentration as a result of a change in water velocity compared to a tracer (left). Nitrite, 1,2DNG and fraction of nitrogen recovered in the form of 1,2 DNG and nitrite (right)	46
Figure 5.2. Nitroester concentration in column effluent resulting from degradation in sorptive soil. Data represent the average obtained from two columns and bars represent the range	50
Figure 5.3. NG degradation in Potomac River sediment. Left: Comparison of degradation by native microbial community and inoculation with JBH1. Right: Modeling results	51
Figure 5.4. Effect of pH (blue line) and NG concentration (red line) on NG degradation rate	52
Figure 5.5. NG degradation after 48 hours after inoculation with JBH1 in the presence of common explosive co-contaminants	54
Figure A-1. Representation of DNAPL spill in porous medium	60
Figure B-1. Hydrolytic degradation of NG as a function of time at various pH values. (data represent average $\pm$ standard of triplicates)	69
Figure B-2. NG relative concentrations as a function of pH after 48 hours. Data represent the average $\pm$ the range	69
Figure C-1. Size distribution analysis	72

Figure D-1. Nitrogen mass balance during NG biodegradation by mixed culture. Data represent average of replicates $\pm$ range	78
Figure D-2. NG concentration and growth during NG biodegradation	79
Figure D-3. TSA plates from NG enrichment prior to isolation of <i>Arthrobacter</i> JBH1	81
Figure D-4. Early microbial enrichment capable of growing on NG (solid lines) at different initial NG concentrations and microbial enrichment after 8 months (dashed lines)	81
Figure E-1. Lineweaver-Burk plot of NG transformation by cell extracts from JBH1 and NADPH (200 $\mu$ M)	86
Figure E-2. Experimental results for initial biodegradation rates associated to initial substrate concentrations fitted to Andrew's model	88
Figure E-3. Modeled NG biodegradation results using the predicted $k$ , $K_M$ , and $K_I$ values (lines) compared with experimental results (markers)	90
Figure E-4. Initial NG degradation rate as a function of pH for <i>Arthrobacter</i> JBH1. Comparison of experimental (markers) v. modeled results (solid line)	91
Figure E-5. Comparison of experimental (markers) versus modeled results (solid line) using substrate inhibition models and including pH effect on degradation. Green: NG concentration, mM. Blue: biomass concentration (mg/L). Yellow: Nitrite concentration (mM). Red: Carbon dioxide (mM)	92

## PROJECT SUMMARY

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Nitroglycerin (NG) is a toxic explosive commonly found in soil and contaminated groundwater at old manufacturing plants and military ranges. When NG enters an aquifer, it behaves as a dense non-aqueous phase liquid (DNAPL). Nitroglycerin is an impact sensitive explosive and therefore excavating the area to remove or treat the contaminant can be dangerous. *In situ* bioremediation and natural attenuation of NG have been proposed as remediation alternatives and it is therefore necessary to understand the degradation mechanisms of NG in contaminated soil and groundwater and investigate the potential for using bioremediation at contaminated sites.

Many bacteria have been isolated for the ability to transform NG as a source of nitrogen, but no isolates have used NG as a sole source of carbon, nitrogen, and energy. We isolated *Arthrobacter* JBH1 from NG contaminated soil by selective enrichment with NG as the sole growth substrate. The degradation pathway involves a sequential denitration to 1,2-dinitroglycerin (DNG) and 1-mononitroglycerin (MNG) with simultaneous release of nitrite.

Flavoproteins of the Old Yellow Enzyme (OYE) family capable of removing the first and second nitro groups from NG have been studied in the past and we identified an OYE homolog in JBH1 capable of selectively producing the 1 MNG intermediate. To our knowledge, there is no previous report on enzymes capable transforming MNG. Here we show evidence that a glycerol kinase homolog in JBH1 is capable of transforming 1 MNG into 1-nitro-3-phosphoglycerol, which could be later introduced into a widespread pathway, where the last nitro group is removed. Overall, NG is converted to CO<sub>2</sub> and biomass and some of the nitrite released during denitration is incorporated into biomass

as well. As a result, NG can be now considered a growth substrate, which changes the potential to bioremediate NG contaminated sites.

The magnitude of the effect of biodegradation processes in the fate of NG in porous systems was unknown, and we have been able to quantify these effects, determine degradation rates, and have evidence that bioaugmentation with *Arthrobacter* sp. strain JBH1 could result in complete mineralization in contaminated soil and sediments contaminated with NG, without the addition of other carbon sources.

Site specific conditions have the potential to affect NG degradation rates *in situ*. Experiments were conducted to investigate NG degradation at various pH values and NG concentrations, and the effects of common co-contaminants on NG degradation rates. *Arthrobacter* JBH1 was capable of growing on NG at pH values as low as 5.1 and NG concentrations as high as 1.2 mM. The presence of explosive co-contaminants at the site such as trinitrotoluene and 2,4-dinitrotoluene lowered NG degradation rates, and could potentially result in NG recalcitrance.

Collectively, these results provide the basis for NG bioremediation and natural attenuation at sites contaminated with NG without the addition of other sources of carbon. Nonetheless, careful attention should be paid to site-specific conditions that can affect degradation rates.

## CHAPTER 1: INTRODUCTION

---

Nitroglycerin (NG) was discovered in 1847 by the Italian chemist Asconio Sobrero during his doctoral studies (86). NG is a liquid at standard temperature and pressure with solubility values ranging between 1.27 and 2 g/L at temperatures ranging between 15 and 25°C (48) and a melting point of 13°C (31). Exposure to NG can be harmful both to wildlife and humans. Exposure effects include headaches, vomiting, decrease in blood pressure, fainting, and cyanosis (10).

Nitroglycerin is a very powerful impact sensitive explosive, and is very difficult to transport and handle in its pure form (31). Alfred Nobel, a fellow student of Sobrero at the University of Turin, tried for many years to find safer ways to produce and handle NG. After trying many different absorbent materials, Nobel found that diatomaceous earth was an inert material capable of absorbing up to 4 times its weight in NG. This mixture (75 % NG, 25 % diatomaceous earth) was a stable, easy to handle explosive, and he later named it dynamite (86). For many years however, there were problems associated with the high freezing point of NG (13.5°C) which made it difficult to use during the winter months. Additionally, thawing of NG is a dangerous process which can result in explosion (31). Antifreeze for dynamite was extensively researched during the first part of the twentieth century and dinitrotoluenes (DNTs) and trinitrotoluene (TNT) were identified as antifreeze substances which do not greatly reduce the explosive power of NG. However, these two compounds are not very effective at extremely low temperatures (31) and in 1907 glycol esters, such as ethylene glycol dinitrate, were introduced as antifreeze substances, which later became an important part of dynamites. In addition to the manufacture of dynamite, NG has been widely used for the production

of propellants and smokeless powder in which it is commonly mixed with nitrocellulose, DNT, and other chemicals (28).

Due to improper handling, release, and disposal to the environment NG has been found in contaminated soil and groundwater samples at various sites, including the Lone Star Army Ammunition Plant, the March Air Force Base (29), the Naval Surface Warfare Station, MD, the Massachusetts Military Reservation (75) and at an abandoned NG manufacturing plant in Somerset West, South Africa (51). Due to its health effects and its explosive nature NG contamination in soils and groundwater poses a risk that requires remedial action (10).

NG behaves as a DNAPL when it enters the subsurface environment and therefore remediation of soil and groundwater contaminated with NG presents unique challenges (90). There are techniques that are used for the remediation of soils and groundwater contaminated with explosives (80). These techniques include excavation followed by composting and incineration (in the case of soils, but is mostly applicable for explosives which are solids at room temperature and remain on the soil surface after a spill, such as TNT or DNT) or pump and treat with carbon sorption (in the case of groundwater). However, pump and treat is known for having high operational costs, and if used for the remediation of a NG plume, it would present potential risks associated with drilling into an NG DNAPL, which could result in an explosion produced because NG is a shock explosive (54). For this reason, approaches where NG is treated *in situ* are attractive. Monitored natural attenuation and *in situ* bioremediation have been used as remediation mechanisms in soils contaminated with other explosives (80), although there is no

evidence that these approaches are viable for soils contaminated with NG, due to its behavior as a DNAPL.

There are many mechanisms that can be involved in natural attenuation of nitroglycerin in contaminated soil and groundwater. These include adsorption to the solid media, dissolution of the DNAPL into the aqueous phase, advection, hydrodynamic dispersion, and diffusion, as well as hydrolysis, and biological degradation (90). The hydrolytic degradation of NG has been studied in the past and it has been determined that it is possible at pH values above 9 in relatively short periods of time (15, 55). As hydrolytic degradation rates are first order with respect to  $\text{OH}^-$  concentration (15), at lower pH values the rate of hydrolytic degradation greatly decreases and is not expected to be a major degradation mechanism in NG contaminated aquifers where pH can be relatively low (the NG manufacturing process uses large quantities of sulfuric and nitric acids).

Biological transformation of NG has been studied in some detail, although most studies have focused on its application in wastewater treatment systems (2, 10, 25, 89). In most cases, these studies have investigated NG as a source of nitrogen in the presence of overwhelming carbon and energy sources (10, 51, 53, 89). Under such conditions, NG undergoes a sequential denitration pathway in which NG is transformed to 1,2 or 1,3-dinitroglycerin (DNG), followed by 1 or 2-mononitroglycerin (MNG) and then glycerol, under both aerobic and anaerobic conditions (10, 25, 51, 53, 89). The enzymes involved in denitration have been characterized in some detail (13, 50, 78, 91).

Growth on NG was previously observed under aerobic conditions by a mixed culture originating from activated sludge (2), although there are some uncertainties

regarding the very low increase in Optical Density (OD) resulting from NG transformation and the potential for residual organic carbon from activated sludge during the enrichments. However, no pure culture capable of growing on NG as the sole source of carbon and nitrogen is previously known. It is hypothesized the existence of such culture would provide evidence that supports the applicability of monitored natural attenuation or approaches that include bioremediation of NG contaminated sites which often lack other easily degradable carbon and nitrogen sources.

Results from a recent study using columns packed with soil (20) showed a decrease in NG concentrations when no biocidal agent was added (not observed in the presence of biocide), suggesting the possibility that NG degradation is occurring in porous medium. However, NG concentrations used in this study were very low (1 mg/L) and no mass balances or biodegradation indicators were used, leaving many questions regarding the biodegradation of NG in porous medium unanswered: Are NG biodegradation rates in porous medium high enough for bioremediation to be considered a major treatment mechanism? What are the biodegradation indicators that can be used in the field? What are the necessary conditions for NG biodegradation?

The principal objective of the research project presented in this document was to investigate if nitroglycerin can be biodegraded in the absence of alternative sources of carbon and nitrogen. This document begins with a literature review (Chapter 2) aimed at providing the reader with background information on the physicochemical characteristics of NG, the extent of NG contamination, and literature available regarding the transformation of NG when used as a source of nitrogen source.



Chapters 3, 4, and 5 describe the results of laboratory experiments designed to address the following specific research objectives:

1. Isolate a pure culture capable of growing on NG as the sole source of carbon and nitrogen (Chapter 3)
2. Investigate the NG degradation pathway when NG is used as a source of carbon and nitrogen and characterize the enzymes involved in this transformation (Chapter 4)
3. Evaluate the ability of microorganism to degrade NG in porous media and identify the necessary conditions, the bottlenecks, and biodegradation indicators that can be used in the field to recognize the degradation of NG (Chapter 5)

Chapter 6 summarizes the results presented in chapters 3, 4, and 5, and identifies several questions remaining that should be addressed in the future. Additionally, it describes the engineering significance of this project.

Appendixes A-E describe theoretical considerations regarding the mobilization of NG in the subsurface and present laboratory results aimed at studying:

- Hydrolytic transformation of NG as a function of pH
- Characteristics of soils described in Chapter 5
- The enrichment used to isolate *Arthrobacter* sp. strain JBH1
- Kinetic considerations associated with pH and substrate inhibition

## CHAPTER 2: LITERATURE REVIEW

Nitroglycerin or glycerol trinitrate ( $\text{C}_3\text{H}_5(\text{ONO}_2)_3$ , molecular weight 227.09 g/mol, is composed of three nitroester bonds attached to a glycerol backbone (Figure 2.1). It is a viscous colorless oil at standard temperature and pressure (55) with a melting point of  $13.5^\circ\text{C}$  and boiling point of  $250^\circ\text{C}$  (85). Water solubility of this compound ranges between 1.25 and 1.95 g/L at temperatures between  $20^\circ\text{C}$  and  $25^\circ\text{C}$  (14, 55, 59, 73, 87, 97). The vapor pressure ranges between  $3.8 \times 10^{-4}$  mm Hg ( $20^\circ\text{C}$ ) (81) and  $1.8 \times 10^{-3}$  mm Hg ( $25^\circ\text{C}$ ) (40) with an estimated Henry's constant of  $1 \times 10^{-6}$  (dimensionless) (55, 60), which indicates the compound's tendency to remain in the aqueous phase.

NG is prepared by nitration of glycerin (99% purity). Nitration is usually conducted at  $25^\circ\text{C}$  (or below) by adding glycerin slowly to an agitated acid mixture containing approximately 40% nitric acid and 60% sulfuric acid (31) (Figure 2.1).

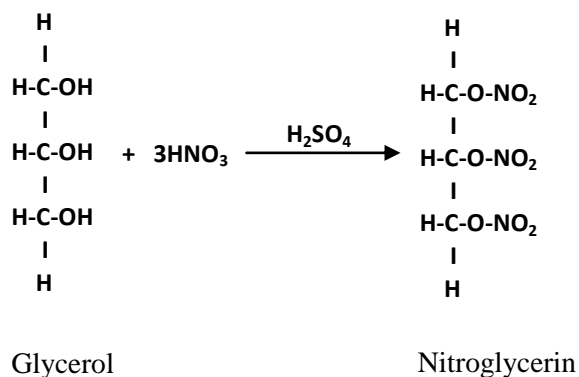


Figure 2.1. Nitration of glycerol to produce nitroglycerin

NG is a very powerful and impact sensitive explosive with a detonation rate of 1600-1900 m/s in glass tube (7700 in steel tube) and reaches an explosion temperature of

222°C in 5 seconds (31). This compound can be readily absorbed through the skin and lungs. Nitroglycerin is a vasodilator which affects the cardiovascular system, blood, and nervous system in humans (38). It is believed that in the human body, bioactivation resulting in vasodilating activity involves the esterase activity of ALDH-2 yielding 1,2-DNG and nitrite, which is further bioactivated to vasodilating species. ALDH-2 possesses two different enzymatic activities. The dehydrogenase activity catalyzes the conversion of aldehydes to carbonic acids using  $\text{NAD}^+$  as a cofactor. The esterase activity catalyzes the hydrolysis of esters to the free acid and an alcohol without requiring a cofactor) (26). Exposure to NG can cause headaches, nausea, vomiting, and lightheadedness, although convulsions, paralysis, impaired vision, breathing difficulties, and circulatory collapse, or death are also possible depending on the dose (10, 49).

## 2.1 NITROGLYCERIN CONTAMINATION

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Due to poor management and handling, NG contamination of soil and groundwater has been detected at several sites. Sites include mostly military bases and firing ranges, although contamination at former manufacturing sites has also been reported. Table 2.1 lists some of the sites that have reported NG contamination in the past.

In the United States, most of the soil contaminated with explosives is treated by excavation followed by composting or incineration (80). Treatment of groundwater contaminated with explosives is primarily conducted by pump and treat with carbon sorption. In addition, some research has been conducted on the *in situ* bioremediation and

natural attenuation of some explosives and production intermediates, such as trinitrotoluene and dinitrotoluene (34, 80, 83, 99). However, the bioremediation and natural attenuation of NG is not well understood, and although some research has been conducted to study the biological transformation of NG, most studies have focused on its degradation in wastewater and little is known about its degradation in soil and groundwater.

**Table 2.1. NG contamination at different sites**

Site	Concentration	Reference
Yakima Firing Range	0.4 to 6.6 mg /kg	(61)
Wellington Antitank Rocket Range	8.0 to 110 mg/kg	(62)
Massachusetts Military Reservation	up to 91.7 mg/kg	(75)
Former March Air Force Base	Not reported	(1)
DuPont, Barksdale	2.5 µg/L (groundwater)	(57)
Camp Edwards, Massachusetts	up to 69.6 mg/kg	(20)

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## 2.2 BIOLOGICAL DEGRADATION OF NITROGLYCERIN

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Several groups have investigated the biological transformation of nitroglycerin under aerobic and anaerobic conditions, with pure and mixed cultures, and mostly in the presence of additional sources of carbon. Table 2.2 summarizes research conducted to date that studies the microbial transformation of NG.

All recent publications converge to a single denitration pathway for conditions in which NG is used as a nitrogen source, both for aerobic and anaerobic conditions. First, one nitro group is reduced from the nitroglycerin molecule, converting it to one of the

isomers 1,3-DNG or 1,2-DNG. A second nitro group is removed converting the molecule to 1-GMN or 2-GMN (2, 10, 24, 89). The removal of the last nitro group to obtain glycerol is always more difficult, but can also be achieved under aerobic conditions (51, 53). Even under reduction utilizing elemental iron, a similar chain of intermediates and final products was observed, with nitro groups being further reduced to ammonia groups (58). The pathway through which nitroglycerin is transformed to glycerol in the presence of additional sources of carbon is shown in Figure 2.2 (24, 51, 53, 58, 89).

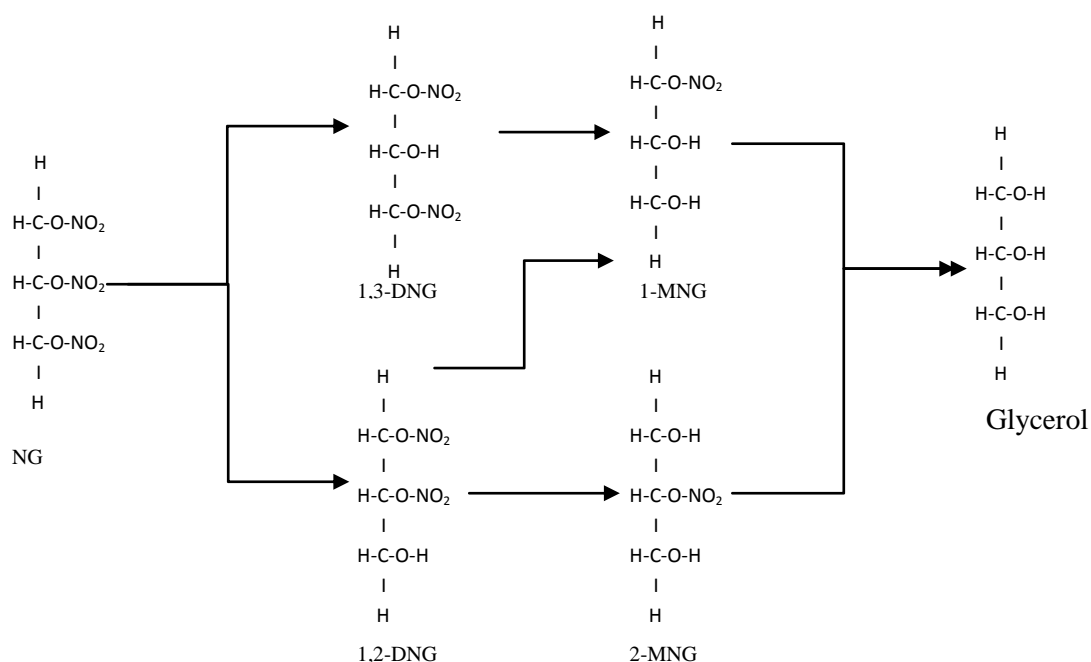


Figure 2.2. Nitroglycerin biological transformation pathway when used as a nitrogen source

Enzymes capable of removing the first and second nitro-groups from NG are all flavin-containing enzymes, members of the Old Yellow Enzyme (OYE) family. Five different enzymes capable of such denitration have been studied in the past and some of their crystal structures are now available (12, 13, 32, 50, 78, 91, 92). This transformation

requires the addition of one NAD(P)H molecule and produces one nitrite molecule for each nitro group removed from NG. Previous reports indicate that these enzymes are regioselective, producing different ratios of degradation intermediates. The specific physiological role of OYEs is still unknown, although based on the broad substrate specificity of several members of this family it is possible that these enzymes do not have a single physiological substrate in vivo (32). Instead, it is believed that this family of enzymes is involved in general stress response, helping to maintain the redox state of the cell.

Until recent years it was believed that nitrate esters were only of anthropogenic origin and therefore understanding the capability of microbes of transforming these compounds was a challenge. Recent research however, showed evidence of high concentrations of methyl and ethyl nitrate in sea water and air samples along two Atlantic Ocean transects, providing the first direct evidence of a natural source of these compounds (19). This observation offers an initial step to understanding how the transformation of nitroesters evolved. Nonetheless, there are still many questions regarding the complete denitration of NG.

**Table 2.2 Summary of resented publications that investigate the microbial degradation of NG**

Author (s)	Description	Use of co-substrate	Source of inoculum	Medium	Enzymes
Smets et al. (77)	Thermodynamic analysis to investigate if complete mineralization of nitroglycerine is possible. Authors assume initial nitro-group removal to obtain glycerol and then glycerol is used as a source of carbon (energy source). Findings suggest complete mineralization is feasible under aerobic and anaerobic conditions. <ul style="list-style-type: none"> <li>Only theoretical</li> </ul>	No	n/a	n/a	n/a
Meng et al. (53)	Microorganisms were selected from soil /sediment samples known to have been exposed to NG. <i>Bacillus thuringiensis/cereus</i> and <i>Enterobacter agglomerans</i> were isolated (most effective for transforming NG). For both isolates denitration activities were expressed constitutively (NG not required for induction). Analytical tools used showed denitration pathway to be sequential denitration. Complete conversion to glycerol after long term incubation of cell extracts. <ul style="list-style-type: none"> <li>Cells selected based on their ability to use NG as only nitrogen source and ability to survive under high (toxic) NG concentrations (concentration not mentioned)</li> <li>Aerobic conditions</li> <li>Use glucose and yeast extracts for cultivation</li> <li>Use NG concentrations as high as 2.5mM</li> <li>Rates of 0.07 to 0.1 mmol/g of cell per h</li> </ul>	<ul style="list-style-type: none"> <li>Glucose</li> <li>Yeast extract</li> </ul>	Soil/sediment samples	Liquid	n/a
White et al. (89)	<i>Agrobacterium radiobacter</i> was isolated under aerobic and nitrogen limiting conditions (isolates from soil, river water and activated sewage sludge). NG was only degraded to glycerol mononitrates (mononitrates were not biodegraded). Denitration pathway same as previous studies. Observations <ul style="list-style-type: none"> <li>Complete denitration was not achieved</li> <li>Aerobic conditions</li> </ul>	<ul style="list-style-type: none"> <li>Glycerol</li> </ul>	Sewage	Liquid	n/a

**Table 2.2 Summary of recent publications that investigate the microbial degradation of NG (continued)**

Author (s)	Description	Use of co-substrate	Source of inoculum	Medium	Enzymes
Christodoulatus et al. (24)	Used anaerobic conditions. First denitration to glycerol dinitrate, glycerol mononitrate, and then they hypothesize that a utilizable carbon source is produced, although they do not address the fact that digester sludge may contain other sources of carbon. Addition of co-substrates substantially increased rates of conversion. Observations: <ul style="list-style-type: none"> <li>Anaerobic</li> <li>Time for degradation with co-substrate added is in the 20-40 day range (Depending on the co-substrate concentration), whereas time for degradation without the addition of co-substrate is around 110 days.</li> </ul>	Glucose (Not for all experiments)	Digester sludge	Liquid	n/a
Bhaumik et al. (10)	Used batch and packed bed bioreactors to investigate aerobic and anaerobic biodegradation of NG. Observations <ul style="list-style-type: none"> <li>Aerobic: mixed cultures and <i>Phanerochaete chrysosporium</i></li> <li>Anaerobic: anaerobic digester sludge</li> <li>Showed complete denitration</li> <li>Rate of bioconversion was dependent on co-substrate concentration</li> </ul>	Glucose	<ul style="list-style-type: none"> <li><i>Phanerochaete chrysosporium</i> from the American Type Culture collection (ATCC)</li> <li>Activated sludge</li> </ul>	Packed bed (1/6" plastic flakes) reactor	n/a
Accashian et al. (2)	Mixed microbial cultures from aeration tank previously exposed to NG. No external carbon source was added. Denitration resulted in release of nitrite (reductive denitration mechanism). Toxicity for concentrations above 0.3mM. Maximum specific growth rate of $0.048 \pm 0.005 \text{h}^{-1}$ . Observations: <ul style="list-style-type: none"> <li>Results for nitrite concentrations are lower than expected using stoichiometric relationships</li> <li>Change in OD was too low based on observed NG degradation</li> </ul>	NG is used as only C source * Glycerol is added to culture media as required.	Mixed liquor from wastewater treatment plant	Liquid	n/a



**Table 2.2 Summary of resented publications that investigate the microbial degradation of NG (continued)**

Author (s)	Description	Use of co-substrate	Source of inoculum	Medium	Enzymes
Marshall et al. (51)	<p>4 bacteria species were isolated from soil samples taken from washwater soakaway at disused NG manufacturing plant.</p> <ul style="list-style-type: none"> <li>• <i>Pseudomonas putida</i></li> <li>• <i>Arthrobacter</i></li> <li>• <i>Klebsiela</i></li> <li>• <i>Rhodococcus</i></li> </ul> <p>Utilize nitroglycerin as sole nitrogen source removing nitro groups sequentially to obtain glycerol mononitrate (<i>Arthrobacter</i>) was only able to remove the first nitro group. <i>Rhodococcus</i> was able to transform MNG.</p> <p>Observations:</p> <ul style="list-style-type: none"> <li>• No nitrite quantification. Unknown if glycerol was final product or if a different nitrated intermediate accumulated.</li> </ul>	Glycerol	Soil samples	Liquid	n/a
Servent et al. (74)	<p>Phanerochaete chrysosporium was used to study NG degradation pathway using 14C-labeled substrate. Evidence for a multienzymatic system resulting in DNG and MNG. Several independent enzymatic activities detected</p> <ul style="list-style-type: none"> <li>• Aerobic glutathione S-transferase activity</li> <li>• Anaerobic NADPH-dependent soluble cytochrome P450-like activity</li> <li>• Enzymatic activities dependent upon the presence of NADPH or ferrous ions</li> </ul>	n/a	n/a	Liquid	n/a
Zhang et al. (100)	<p><i>Penicillium corylophilum</i> Dierckx able to completely denitrate NG in buffered medium with glucose and ammonium nitrate 48 µmol of NG was transformed stepwise to DNG (48 hours) and MNG (within 168 hours). Denitration of MNG was achieved in 336 hours.</p> <ul style="list-style-type: none"> <li>• After 336 hours it is possible that MNG disappearance is due to hydrolysis</li> </ul>	Glucose	Water	Liquid	n/a

**Table 2.2 Summary of resented publications that investigate the microbial degradation of NG (continued)**

Author (s)	Description	Use of co-substrate	Source of inoculum	Medium	Enzymes
Blehert et al. (13)	Investigated NG denitration by purified enzymes from <i>Pseudomonas putida</i> II-B (XenA) and <i>Pseudomonas fluorescens</i> I-C (XenB). Results obtained using XenA showed formation of both DNG isomers. However, after reaching a maximum, the concentration of the 1,2 DNG isomer started decreasing and both MNG isomers accumulated and did not disappear. Results obtained using XenB indicated most of the NG was converted to 1,3 DNG which was not further denitrated and some went to 1,2 DNG, which was further denitrated to 1 MNG which accumulated. No 2 MNG production was observed. No complete denitration was observed in either case. Found several other strains capable of incomplete denitration of NG at low concentrations (0.44 mM) in the presence of glucose including <i>P. aeruginosa</i> JB2, <i>Escherichia coli</i> 8008, <i>Klebsiella oxytoca</i> 8701, <i>K. planticola</i> , <i>E. coli</i> 8101, <i>P. fluorescens</i> , <i>K. oxytoca</i> 8408, <i>P. aerofaciens</i> , <i>P. aerofaciens</i> C16, <i>P. fluorescens</i> 2-79.	Glucose	Soil	Liquid	XenA (3L5L_A GI:291463635) XenB (AAF02539.1 GI:6049283) Flavoproteins Old Yellow Enzyme family. Prefer NADPH over NADH
14 Blehert et al. (12)	XenA and XenB were purified and cloned. <i>XenA</i> encodes a NAD(P)H-dependent flavoprotein that removes either the terminal or the central nitro group from NG but cannot reduce 2,4,6-trinitrotoluene (TNT). <i>XenB</i> encodes a NAD(P)H-dependent flavoprotein that shows fivefold regioselectivity for the removal of the central nitro group from NG and can transform TNT. Expression of <i>xenA</i> and <i>xenB</i> was demonstrated in <i>Escherichia coli</i> DH5 $\alpha$ .	n/a	Soil	Liquid	XenA, XenB
Marshall et al. (50)	Experiments were conducted with purified NerA. After denaturation, NerA liberated FMN. His178, Asn181, and Tyr 183 are closed to FMN in the active site.	n/a	n/a	Liquid	NerA-FMN dependent

**Table 2.2 Summary of resented publications that investigate the microbial degradation of NG (continued)**

Author (s)	Description	Use of co-substrate	Source of inoculum	Medium	Enzymes
French et al. (35)	Isolated <i>Enterobacter cloacae</i> PB2 on the basis of its ability to use nitrate esters such as pentaerythritol tetranitrate and NG as nitrogen sources. This strain contains a soluble PETN reductase capable of denitrating nitrate esters with oxidation of NADPH. PETN reductase (onr) is a monomeric flavoprotein that follows a ping-pong mechanism with competitive substrate inhibition by NADPH and is strongly inhibited by steroids	n/a	n/a	Liquid	Onr (U68759) Flavoprotein, OYE, NADPH dependent
Fitzpatrick et al. (32)	Cloned YqjM from <i>Bacillus subtilis</i> (gram positive) into <i>E. coli</i> . Enzyme was purified and tested for NG transformation. Enzyme is member of OYE family, prefers NADPH over NADH. OYEs are identified as enzymes that are induced as a response of oxidative stress and the presence of toxic xenobiotics and the report suggest that the physiological role of OYEs may rely on the stress response.	n/a	Soil	Liquid	YqjM (P54550), Flavoprotein, OYE. Prefers NADPH over NADH
Clausen et al. (20)	Investigated the sorption of NG to soil medium using column and batch experiments. Some of the experiments were conducted with biocide and some without and sorption parameters were later compared. Differences in $K_d$ values were attributed to microbial degradation of NG. Low NG concentrations were utilized throughout (1 mg/L) and report indicated no NG degradation intermediates were observed at the column's effluent in any of the runs. A single run using higher NG concentration (100 mg/L) did result in NG and NG degradation intermediates at the columns effluent.	No	Soil	Soil columns	n/a
Yost (98)	Used soil reactors (18:1) water to soil ratio to investigate the effect of pH and redox potential on NG biodegradation. Results indicated that degradation is much slower during anaerobic conditions and that degradation rates decrease at lower pH values (investigated pH of 6, 7 and 8).	Glucose	Soil (surface and aquifer)	Stirred soil reactors	n/a

### CHAPTER 3: GROWTH OF *ARTHROBACTER* SP. STRAIN JBH1 ON NITROGLYCERIN AS THE SOLE SOURCE OF CARBON AND NITROGEN

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## Growth of *Arthrobacter* sp. Strain JBH1 on Nitroglycerin as the Sole Source of Carbon and Nitrogen<sup>†</sup>

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***Arthrobacter* sp. strain JBH1 was isolated from nitroglycerin-contaminated soil by selective enrichment. Detection of transient intermediates and simultaneous adaptation studies with potential intermediates indicated that the degradation pathway involves the conversion of nitroglycerin to glycerol via 1,2-dinitroglycerin and 1-mononitroglycerin, with concomitant release of nitrite. Glycerol then serves as the source of carbon and energy.**

Nitroglycerin (NG) is manufactured widely for use as an explosive and a pharmaceutical vasodilator. It has been found as a contaminant in soil and groundwater (7, 9). Due to NG's health effects as well as its highly explosive nature, NG contamination in soils and groundwater poses a concern that requires remedial action (3). Natural attenuation and *in situ* bioremediation have been used for remediation in soils contaminated with certain other explosives (16), but the mineralization of NG in soil and groundwater has not been reported.

To date, no pure cultures able to grow on NG as the sole carbon, energy, and nitrogen source have been isolated. Accashian et al. (1) observed growth associated with the degradation of NG under aerobic conditions by a mixed culture originating from activated sludge. The use of NG as a source of nitrogen has been studied in mixed and pure cultures during growth on alternative sources of carbon and energy (3, 9, 11, 20). Under such conditions, NG undergoes a sequential denitration pathway in which NG is transformed to 1,2-dinitroglycerin (1,2DNG) or 1,3DNG followed by 1-mononitroglycerin (1MNG) or 2MNG and then glycerol, under both aerobic and anaerobic conditions (3, 6, 9, 11, 20), and the enzymes involved in denitration have been characterized in some detail (4, 8, 15, 21). Pure cultures capable of completely denitrating NG as a source of nitrogen when provided additional sources of carbon include *Bacillus thuringiensis/cereus* and *Enterobacter agglomerans* (11) and a *Rhodococcus* species (8, 9). Cultures capable of incomplete denitration to MNG in the presence of additional carbon sources were identified as *Pseudomonas putida*, *Pseudomonas fluorescens* (4), an *Arthrobacter* species, a *Klebsiella* species (8, 9), and *Agrobacterium radiobacter* (20).

Here we describe the isolation of bacteria able to degrade NG as the sole source of carbon, nitrogen, and energy. The inoculum for selective enrichment was soil historically contaminated with NG obtained at a facility that formerly manufactured explosives located in the northeastern United States. The enrichment medium consisted of minimal medium prepared as

previously described (17) supplemented with NG (0.26 mM), which was synthesized as previously described (18). During enrichment, samples of the inoculum (optical density at 600 nm [OD<sub>600</sub>] ~ 0.03) were diluted 1/16 in fresh enrichment medium every 2 to 3 weeks. Isolates were obtained by dilution to extinction in NG-supplemented minimal medium. Cultures were grown under aerobic conditions in minimal medium at pH 7.2 and 23°C or in tryptic soy agar (TSA; 1/4 strength).

Early stages of enrichment cultures required extended incubation with lag phases of over 200 h and exhibited slow degradation of NG (less than 1  $\mu$ mol substrate/mg protein/h). After a number of transfers over 8 months, the degradation rates increased substantially (2.2  $\mu$ mol substrate/mg protein/h). A pure culture capable of growth on NG was identified based on 16S rRNA gene analysis (504 bp) as an *Arthrobacter* species with 99.5% similarity to *Arthrobacter pascens* (GenBank accession no. GU246730). Purity of the cultures was confirmed microscopically and by formation of a single colony type on TSA plates. 16S gene sequencing and identification were done by MIDI Labs (Newark, DE) and SeqWright DNA Technology Services (Houston, TX). The *Arthrobacter* cells stained primarily as Gram-negative rods with a small number of Gram-positive cocci (data not shown); Gram variability is also a characteristic of the closely related *Arthrobacter globiformis* (2, 19). The optimum growth temperature is 30°C, and the optimum pH is 7.2. Higher pH values were not investigated because NG begins to undergo hydrolysis above pH 7.5 (data not shown). The isolated culture can grow on glycerol, acetate,

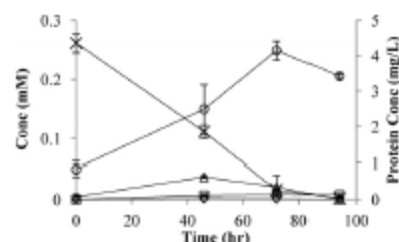


FIG. 1. Growth of strain JBH1 on NG. ×, NG; △, 1,2DNG; ◇, 1MNG; □, 2MNG; ○, protein.

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TABLE 1. Nitrogen mass balance

Time (h)	% of total initial nitrogen by mass recovered in <sup>a</sup> :								Total recovery (%)
	1MNG	2MNG	1,2DNG	1,3DNG	NG	Protein	Nitrite	Nitrate	
0	ND <sup>b</sup>	ND	0.9 ± 0.7	0.8 ± 0.6	82 ± 5.2	0.8 ± 0.2	14 ± 0.7	0.8 ± 0.3	100 ± 5.3
46	0.1 ± 0.0	0.8 ± 0.2	7.9 ± 0.4	ND	35 ± 3.6	2.0 ± 0.5	49 ± 1.1	1.7 ± 0.0	96 ± 4.2
72	0.1 ± 0.0	0.9 ± 0.2	4.3 ± 4.2	ND	5.0 ± 0.4	3.3 ± 0.2	81 ± 4.2	3.9 ± 1.9	98 ± 6.8
94	ND	0.6 ± 0.4	ND	ND	0.6 ± 0.4	3.2 ± 0.0	95 ± 10	2.6 ± 1.6	102 ± 10

<sup>a</sup> Data represent averages of four replicates ± standard deviations.<sup>b</sup> ND, not detected.

succinate, citrate, and lactate, with nitrite as the nitrogen source. Previous authors described an *Arthrobacter* species able to use NG as a nitrogen source in the presence of additional sources of carbon. However, only dinitroesters were formed, and complete mineralization was not achieved (9).

To determine the degradation pathway, cultures of the isolated strain (5 ml of inoculum grown on NG to an OD<sub>600</sub> of 0.3) were grown in minimal medium (100 ml) supplemented with NG at a final concentration of 0.27 mM. Inoculated bottles and abiotic controls were continuously mixed, and NG, 1,2DNG, 1,3DNG, 1MNG, 2MNG, nitrite, nitrate, CO<sub>2</sub>, total protein, and optical density were measured at appropriate intervals. Nitroesters were analyzed with an Agilent high-performance liquid chromatograph (HPLC) equipped with an LC-18 column (250 by 4.6 mm, 5 µm; Supelco) and a UV detector at a wavelength of 214 nm (13). Methanol-water (50%, vol/vol) was used as the mobile phase at a flow rate of 1 ml/min. Nitrite and nitrate were analyzed with an ion chromatograph (IC) equipped with an IonPac AS14A anion-exchange column (Dionex, CA) at a flow rate of 1 ml/min. Carbon dioxide production was measured with a Micro OxyMax respirometer (Columbus Instruments, OH), and total protein was quantified using the Micro BCA protein assay kit (Pierce Biotechnology, IL) according to manufacturer's instructions. During the degradation of NG the 1,2DNG concentration was relatively high at 46 and 72 h (Fig. 1). 1,3DNG, detected only at time zero, resulted from trace impurities in the NG stock solution. Trace amounts of 1MNG appeared transiently, and trace amounts of 2MNG accumulated and did not disappear. Traces of nitrite at time zero were from the inoculum. The concentration of NG in the abiotic control did not change during the experiment (data not shown).

Results from the experiment described above were used to calculate nitrogen and carbon mass balances (Tables 1 and 2). Nitrogen content in protein was approximated using the formula C<sub>5</sub>H<sub>7</sub>O<sub>2</sub>N (14). Because all nitrogen was accounted for

throughout, we conclude that the only nitrogen-containing intermediate compounds are 1,2DNG and 1MNG, which is consistent with previous studies (6, 9, 20). The fact that most of the nitrogen was released as nitrite is consistent with previous reports of denitration catalyzed by reductase enzymes (4, 8, 21). The minor amounts of nitrate observed could be from abiotic hydrolysis (5, 12) or from oxidation of nitrite. Cultures supplemented with glycerol or other carbon sources assimilated all of the nitrite (data not shown).

In a separate experiment cells grown on NG were added to minimal media containing 1,3DNG, 1,2DNG, 1MNG, or 2MNG and degradation over time was measured. 1,2DNG, 1,3DNG, and 1MNG were degraded at rates of 6.5, 3.8, and 8 µmol substrate/mg protein/hour. No degradation of 2MNG was detected (after 250 h), which indicates that 2MNG is not an intermediate in a productive degradation pathway. Because 1,3DNG was not observed at any point during the degradation of NG and its degradation rate is approximately one-half the degradation rate of 1,2DNG, it also seems not to be part of the main NG degradation pathway used by *Arthrobacter* sp. strain JBH1. The above observations indicate that the degradation pathway involves a sequential denitration of NG to 1,2DNG, 1MNG, and then glycerol, which serves as the source of carbon and energy (Fig. 2). The productive degradation pathway differs from that observed by previous authors using both mixed (1, 3, 6) and pure cultures (4, 9, 11, 20), in which both 1,3- and 1,2DNG were intermediates during NG transformation. Additionally, in previous studies both MNG isomers were produced regardless of the ratio of 1,2DNG to 1,3DNG (3, 4, 6, 9, 20). Our results indicate that the enzymes involved in denitration of NG in strain JBH1 are highly specific and catalyze sequential denitrations that do not involve 1,3DNG or 2MNG. Determination of how the specificity avoids misrouting of intermediates will require purification and characterization of the enzyme(s) involved.

Mass balances of carbon and nitrogen were used to de-

TABLE 2. Carbon mass balance

Time (h)	% of total initial carbon by mass recovered in:							Total recovery (%)
	1MNG <sup>a</sup>	2MNG <sup>a</sup>	1,2DNG <sup>a</sup>	1,3DNG <sup>a</sup>	NG <sup>a</sup>	Protein <sup>a</sup>	CO <sub>2</sub> <sup>b</sup>	
0	ND <sup>c</sup>	ND	1.6 ± 1.2	1.9 ± 0.4	92 ± 5.8	4.4 ± 0.9		100 ± 8.4
46	0.5 ± 0.2	2.6 ± 0.6	13 ± 0.7	ND	39 ± 3.9	13 ± 3.0	28 ± 5.7	96 ± 14.1
72	0.4 ± 0.0	2.9 ± 0.7	7.3 ± 7.0	ND	5.6 ± 0.4	22 ± 1.2	59 ± 8.3	97 ± 17.6
94	ND	2.8 ± 0.3	ND	ND	0.8 ± 0.5	18 ± 0.3	71 ± 4.5	93 ± 5.6

<sup>a</sup> Data represent averages of four replicates ± standard deviations.<sup>b</sup> Data represent averages of duplicates ± standard deviations.<sup>c</sup> ND, not detected.

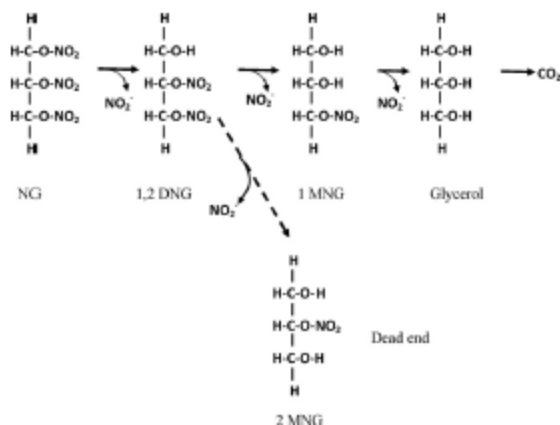


FIG. 2. Proposed NG degradation pathway.

termine the following stoichiometric equation that describes NG mineralization by *Arthrobacter* sp. strain JBH1:  $0.26\text{C}_3\text{H}_5(\text{ONO}_2)_3 + 0.33\text{O}_2 \rightarrow 0.03\text{C}_3\text{H}_7\text{O}_2\text{N} + 0.63\text{CO}_2 + 0.75\text{NO}_2^- + 0.75\text{H}^+ + 0.17\text{H}_2\text{O}$ . The result indicates that most of the NG molecule is being used for energy. The biomass yield is relatively low (0.057 mg protein/mg NG), with an  $f_s$  (fraction of reducing equivalents of electron donor used for protein synthesis) of 0.36 (10), which is low compared to the aerobic degradation of other compounds by pure cultures, for which  $f_s$  ranges between 0.4 and 0.6 (10, 14). The results are consistent with the requirement for relatively large amounts of energy during the initiation of the degradation mechanism (each denitration probably requires 1 mole of NADH or NADPH [21]).

Although NG degradation rates were optimal at pH 7.2, they were still substantial at values as low as 5.1. The results suggest that NG degradation is possible even at low pH values typical of the subsurface at sites where explosives were formerly manufactured or sites where nitrite production lowers the pH.

NG concentrations above 0.5 mM are inhibitory, but degradation was still observed at 1.2 mM (data not shown). The finding that NG can be inhibitory to bacteria at concentrations that are well below the solubility of the compound is consistent with those of Accashian et al. (1) for a mixed culture.

The ability of *Arthrobacter* sp. strain JBH1 to grow on NG as the carbon and nitrogen source provides the basis for a shift in potential strategies for natural attenuation and bioremediation of NG at contaminated sites. The apparent specificity of the denitration steps raises interesting questions about the evolution of the pathway.

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## CHAPTER 4: ENZYMATIC REACTIONS THAT ALLOW *ARTHROBACTER* SP. STRAIN JBH1 TO GROW ON NITROGLYCERIN AS THE SOLE SOURCE OF CARBON AND NITROGEN

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### 4.1 ABSTRACT

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Several Old Yellow Enzymes (OYEs) are capable of transforming nitroglycerin (NG) to dinitro- or mononitroglycerols, and several strains containing such enzymes are capable of using NG as the nitrogen source. However, only recently a pure culture capable of growing on NG as the sole source of carbon and nitrogen was identified (42), and the exact mechanism used by this strain to incorporate NG into a productive pathway is still unknown. Here we show evidence that in *Arthrobacter* sp. strain JBH1 NG is selectively denitrated to 1 mononitroglycerin (1 MNG) by an OYE homolog, and that 1 MNG is later transformed by a glycerol kinase homolog, resulting in 1-nitro-3-phosphoglycerol, which enters central metabolism.

### 4.2 INTRODUCTION

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Many flavoproteins capable of removing the first nitro group from NG (to form dinitroglycerin, DNG) have been previously identified and characterized (50). These enzymes include pentaerythriol tetranitrate reductase (Onr) from *Enterobacter cloacae* (35), NerA from *Agrobacterium radiobacter* (50, 78), YqjM from *Bacillus subtilis* (gram positive) (32), and reductases XenA and XenB from *Pseudomonas putida* and *Pseudomonas fluorescens* (12). All of these enzymes are oxidoreductases, members of the old yellow enzyme family (50, 91), which use preferably NADPH (as supposed to



NADH) as a reducing agent, removing nitro groups from NG and releasing nitrite (13, 34, 50, 91). However, the protein alone cannot catalyze the reaction and therefore the cofactor flavin mononucleotide (FMN) is required for denitration by OYEs (91). In most cases both DNG isomers are produced although selectivity for either C1 or C2 has been observed in some cases (13, 78). Fewer publications refer to the enzymatic processes involved in the removal of the second nitro group, although there is evidence that a single enzyme can remove the first and second nitro groups (13) sequentially, releasing 2 moles of nitrite per mol of NG utilized and consuming one mol of NADPH per mol of nitrite released. In this case both MNG isomers are observed as a result of the denitration process, and in the case of XenA, enzymatic regioselectivity results in a preferential production of 1 MNG as compared to 2 MNG (13). Although there is evidence of the complete conversion of NG to glycerol using bacterial cell extracts (53), there is no information available on the enzymes associated with the removal of the third nitro group (degradation of MNG) and research conducted with purified enzymes suggests that a different enzyme (from that converting NG into MNG) may be required to convert MNG into glycerol *in vivo* (13).

The ability of *Arthrobacter* sp. strain JBH1 (42) to grow on NG as a sole source of carbon, energy, and nitrogen presents a foundation for the study of potential remediation strategies that involve biodegradation and natural attenuation at NG contaminated sites. However, the precise denitration mechanism used by JBH to grow on NG is unknown, and understanding this pathway could answer many questions regarding the recalcitrance of NG intermediates *in situ* and the use of NG as a carbon and nitrogen source. Here we describe the NG denitration mechanism in JBH1 and the genes encoding the enzymes

involved in this pathway. Understanding the MNG degradation pathway is essential to the development of bioremediation or natural attenuation, including methodologies to demonstrate that processes are functioning in either treatment alternative or to enhance biodegradation rates.

#### 4.3 MATERIALS AND METHODS

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*Chemicals.* NG, 1,2 DNG, 1,3 DNG, and 1 MNG standard solutions in acetonitrile were purchased from Cerilliant (Round Rock, TX). Methanol Chromasolv plus for HPLC > 99.9% (Sigma Aldrich, Montana) was used for chromatography; glycerol used for NG manufacturing was spectrophotometric grade > 99.5% and was purchased from Acros Organics. NG was synthesized as previously described (86).

*Analytical.* Nitroglycerin, 1,2DNG, 1,3DNG, 1 MNG, and 2 MNG were quantified using Agilent HPLC equipped with a Supelguard guard column (10 x 4.6 mm, Supelco, Bellefonte, PA), a Supelco LC-18 column (250 x 4.6 mm, 5  $\mu$ m, Supelco) and a UV detector. Methanol-water (50% v/v or 20 % v/v) was used as the mobile phase with a flow rate of 1ml/min and the wavelength was set at 214 nm. Nitrite was qualitatively analyzed using the nitrite colorimetric method 4500-NO<sub>2</sub><sup>-</sup>B (21) and quantitative measurements of nitrite and nitrate were conducted using a Dionex IC equipped with a IonPac AS14A Anion-Exchange Column (Dionex, CA) at a flow rate of 1ml/min. Total protein was quantified using the Micro BCA Protein Assay Kit (Pierce Biotechnology, IL).

*Genome sequencing and annotation.* Genomic DNA from JBH1 grown in LB was isolated using the UltraClean Microbial DNA isolation kit (Mobio Laboratories, Inc., California) following manufacturer's directions. DNA was sent to Emory University (Atlanta, GA) for 454 sequencing and sequencing results were later analyzed using the

CLC genomics workbench (11). The RAST (Rapid Annotation using Subsystem Technology) Prokaryotic Annotation Service (8) was used to annotate the genome and annotation results were used to identify members of the OYE family.

*Fosmid DNA sequencing and in silico analysis.* The CopyControl Fosmid Library Production kit (Epicentre Biotechnologies, Madison, Wisconsin) was used to construct a fosmid library with DNA isolated from JBH1, following manufacturer's instructions. Fosmid pJBH1A3 DNA (Table 4.1) was purified with a FosmidMAX DNA purification kit (Epicentre Biotechnologies, WI) and was sent for 454 sequencing at the Georgia Genomics Facility (Athens, GA). The reads were assembled with CLC Genomics Workbench (11). The RAST (Rapid Annotation using Subsystem Technology) Prokaryotic Annotation Service (8) was used to identify the open reading frames and annotation was later verified using BLAST (4).

*Transposon mutagenesis and screening.* Following manufacturer's instructions the EZ-Tn5™ <oriV/KAN-2> Insertion Kit (Epicentre Biotechnologies, Madison, Wisconsin) was used to conduct transposon mutagenesis of the fosmid clone pJBH1A3 containing genes whose products are able to release nitrite from 1 MNG. Colonies capable of growing on LB agar with chloramphenicol (34 mg/L) and kanamycin (50 mg/L) were transferred to 96-well plates containing LB liquid medium with chloramphenicol (34 mg/L), kanamycin (50 mg/L), and CopyControl fosmid induction solution (Epicentre Biotechnologies, Madison, WI), and placed on a shaker (250 rpm) at 30°C for 16 h. After growth, 50 µL samples from each well were transferred to a second 96 well plate containing 50 µL of 1 MNG (10 mg/L) in phosphate buffer (26 mM) and incubated for 16 hours at room temperature. Fosmid clones were screened

colorimetrically for nitrite release from 1 MNG. Mutants unable to release nitrite from 1 MNG were sequenced with Ez-Tn5 specific outward-reading primers (Ez-Tn5 KAN-2 FP-1 Ez-Tn5 KAN-2 RP-1, Epicentre Biotechnologies, Madison, WI) by Genewiz, NJ.

*Cloning of old yellow enzymes (OYE1, OYE2, OYE3, OYE4) and glycerol kinase (IMNG-GK).* *OYE1, OYE2, OYE3, OYE4* or *IMNG-GK* was amplified by PCR using the corresponding primers (described in Table 1) (Integrated DNA Technologies, Coralville, IA) and the Takara Ex Taq polymerase (Takara Bio USA, Madison, WI). The purified PCR products were ligated into NdeI and Hind III (*OYE1*) or EcoRI and Hind III (*OYE2, OYE3, OYE4*, and *IMNG-GK*) sites of the pET-24a vector (Invitrogen Corp., Carlsbad, CA). The resulting recombinant plasmids, pJBH1-1, pJBH1-2, pJBH1-3, pJBH1-4, and pJBH1-GK, were transformed into *E. coli* DH5 $\alpha$  (New England BioLabs, Ipswich, MA) (Table 1) to maintain the plasmid or into *E. coli* Rosetta 2(DE3) competent cells (Novagen) (Table 6.1) for overexpression according to the manufacturers' instructions.

*Overexpression of OYE1, OYE2, OYE3, OYE4, and IMNG-GK.* Single colonies of *E. coli* Rosetta 2(pJBH1-1), Rosetta 2(pJBH1-2), Rosetta 2(pJBH1-3), Rosetta 2(pJBH1-4), or Rosetta 2(pJBH1-GK) were transferred into LB medium supplemented with kanamycin (50 mg/L) and chloroamphenicol (34 mg/L) and incubated for 3 hours at 37°C with shaking. Isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) (200 mg/L) was added to each culture and cultures were incubated at 37°C with shaking for 12 more hours. Cultures containing putative OYEs were centrifuged at 4°C and triple washed with phosphate buffer (26 mM, pH 7.2). The culture containing the *IMNG-GK* gene was washed with glycine buffer (100 mM, pH 8.3) after centrifugation. Overexpression of the enzymes was verified by protein gel electrophoresis.

*Enzyme assays.* Wild type JBH1 was grown in LB at room temperature for 5 days. JBH1 cells were centrifuged and triple washed with phosphate (26 mM, pH 7.2) or glycine (100 mM, pH 8.3) buffer, depending on the application. Suspended cells (in glycine or phosphate buffer) were passed twice through a French pressure cell at 20,000 lb/in<sup>2</sup>. Cell debris was removed by centrifugation (20,000 × g, 4°C, 60 min). Enzyme assays were conducted at room temperature in phosphate buffer (NG transformation) or glycine buffer (1 MNG transformation). The protein mixtures contained 1.1 to 1.25 mg of protein/ml. JBH1 cell extracts in phosphate buffer (26 mM) were amended with NG (~70 μM) and NADPH (140 μM) was added to the mixture after 10 minutes of incubation. Samples were analyzed for NG disappearance and the formation/ disappearance of the degradation intermediates 1,2 and 1,3 DNG and 1 and 2 MNG by HPLC. In a separate test, JBH1 cell extracts in glycine buffer (100 mM, pH 8.3) were amended with MNG (~70 μM) and incubated for 10 minutes. Following, ATP (100 μM) and MgCl<sub>2</sub> (250 μM) were added to the mixture and again the formation/disappearance of potential intermediates was analyzed by HPLC. After appropriate intervals, HCl (1N) (5% v/v) was added to individual samples to stop the reaction, and the mixture was centrifuged to remove debris prior to chromatography analysis. Cell pellets from Rosetta 2(pJBH1-GK) were suspended glycine buffer (100 mM), passed twice through the French press, and centrifuged to remove cell debris. Cell extracts in glycine buffer (pH 7.6, 8.3, or 9.3) were amended with 1MNG (50, 100 or 150 μM), ATP (100, 200, or 300 μM), MgCl<sub>2</sub> (0, 250, 500, or 750 μM) and ADP (0 or 100 μM). 1MNG disappearance was measured by HPLC and nitrite/nitrate was analyzed by ion chromatography.

*Transformation of NG by whole cells of Rosetta 2(pJBH1-1), Rosetta 2(pJBH1-2), Rosetta 2(pJBH1-3), and Rosetta 2(pJBH1-4). Overexpressed, washed E. coli Rosetta 2(pJBH1-1), Rosetta 2(pJBH1-2), Rosetta 2(pJBH1-3), or Rosetta 2(pJBH1-4) in phosphate buffer (26 mM, pH 7.2) was amended with NG (0.29 mM) and incubated at room temperature for 10 hours. Samples were filter-sterilized prior to analysis of NG and NG transformation products by HPLC.*

**Table 4.1. Bacterial strains, plasmids, and primers used in this study**

Strain, plasmid or primer	Description	Reference or Source
<b>Strains</b>		
<i>Arthrobacter</i> sp. Strain JBH1	Grows on NG as sole source of C and N	(42)
<i>Escherichia coli</i> EPI 300	Host strain for pJBH1A3 and for Tn5 transposon mutants	Epicentre (Madison, WI)
<i>Escherichia coli</i> 5α	<i>fhuA2Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17i</i> ; Host to maintain plasmids pJBH1-1, pJBH1-2, pJBH1-3, pJBH1-4, and pJBH1-GK	New England BioLabs, (Ipswich, MA)
<i>Escherichia coli</i> Rosetta 2 (DE3)	F- ompT hsdSB( $r_B^-$ $m_B^-$ ) gal dem pRARE2 (Cam <sup>R</sup> ). Host to overexpress OYE1, OYE2, OYE3, OYE4, and 1MNG-GK	Novagen
<b>Plasmids</b>		
pCC1FOS	Cam <sup>r</sup> , 8.1-kb fosmid vector for the construction of genomic library	Epicentre (Madison, WI)
pET24a	Kan <sup>r</sup> , 5,310 -bp overexpression vector	Novagen
pJBH1A3	Cam <sup>r</sup> , 41-kb pCC1FOS containing 1MNG-GK from JBH1	This study
pJBH1-1	Kan <sup>r</sup> , 6,378-bp pET-24a containing the OYE1 from <i>Arthrobacter</i> sp. strain JBH1	This study
pJBH1-2	Kan <sup>r</sup> , 6,333-bp pET-24a containing the OYE2 from <i>Arthrobacter</i> sp. strain JBH1	This study
pJBH1-3	Kan <sup>r</sup> , 6,408-bp pET-24a containing the OYE3 from <i>Arthrobacter</i> sp. strain JBH1	This study
pJBH1-4	Kan <sup>r</sup> , 6,567-bp pET-24a containing the OYE4 from <i>Arthrobacter</i> sp. strain JBH1	This study
pJBH1-GK	Kan <sup>r</sup> , 6,804-bp pET-24a containing the 1MNG-GK from <i>Arthrobacter</i> sp. strain JBH1	This study
<b>Primers</b>		
OYE1-F <sup>a</sup>	5' ATA CAT CAT ATG ATG CTG TTT TCC CCGTTG AC 3'	This study
OYE1-R <sup>b</sup>	5' ATATAT AAG CTT TTA GCC CGC GTA CGC 3'	This study
OYE2-F <sup>c</sup>	5' AAC GAA TTC ATG TGC CAG TAC TCC TCG 3'	This study
OYE2-R <sup>b</sup>	5' AAC AAG CTT CTA GTC CCC CTT AGC CTT CAC 3'	This study
OYE3-F <sup>c</sup>	5' AAC GAA TTC GTG CCG GCA CTG TTC CGG 3'	This study
OYE3-R <sup>b</sup>	5' AAC AAG CTT CTA AAA CGA ATG CCG GGG CAC 3'	This study
OYE4-F <sup>c</sup>	5' AAC GAA TTC ATG CCG CAT CTC TTC ACC CCG 3'	This study
OYE4-R <sup>b</sup>	5' AAC AAG CTT TCA TAA GCC GTC AGA GGC ACT C 3'	This study
GK3-F <sup>c</sup>	5' AGA ATA GAA TTC GTA CCA GTG AGC GAC TAC ATC 3'	This study
GK3-R <sup>b</sup>	5' AGA ATA AAG CTT CGC TGT TCA CAA CGT CCC 3'	This study

<sup>a</sup> Engineered NdeI site

<sup>b</sup> Engineered Hind III site

<sup>c</sup> Engineered EcoRI site

## 4.4 RESULTS

### 4.4.1 TRANSFORMATION OF NG TO 1 MNG

Old yellow enzymes are capable of transforming NG in the presence of NADPH.

However, to date there are no reports of OYEs capable of removing the last nitro group that denitrates MNG to glycerol. To investigate if an OYE could be responsible for the denitration of MNG in JBH1, phosphate buffer with NG was amended with JBH1 cell extracts. No significant degradation of NG was observed. After 10 minutes of incubation, NADPH was added to the mixture, resulting in disappearance of NG and concomitant formation of 1,2 DNG and MNG. The concentration of degradation intermediates was stoichiometric to the disappearance of NG (the total nitroester concentration remained constant) (Figure 4.1).

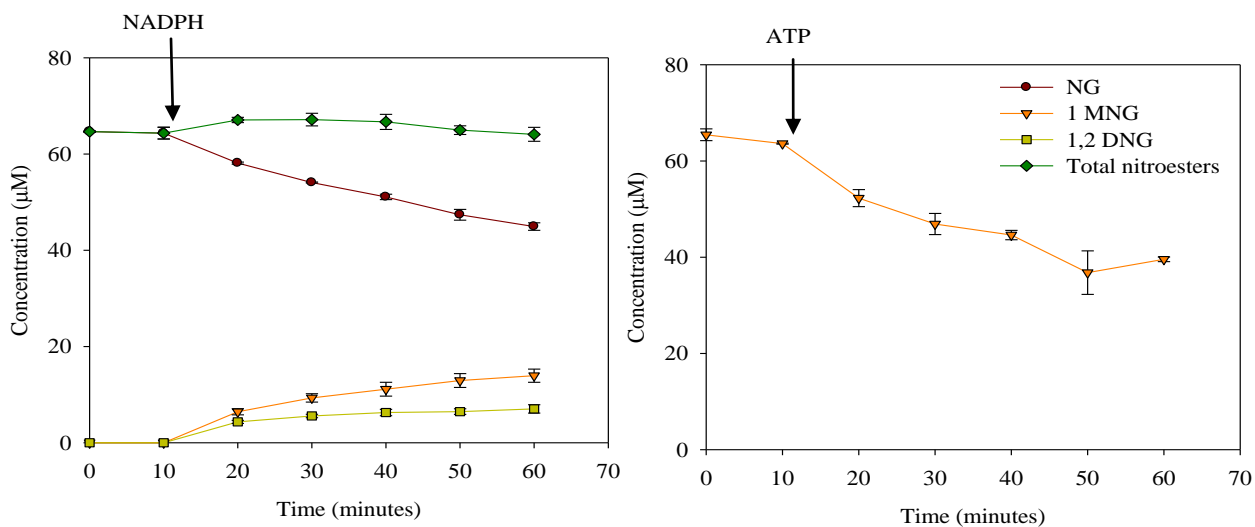


Figure 4.1. NG transformation in cell extracts after addition of NADPH (left) and 1 MNG transformation in cell extracts after addition of Mg-ATP (right)

To identify the gene (s) responsible for the initial denitration of NG to 1 MNG, the genome was sent for DNA sequencing and annotation. Four putative OYEs were detected in the genome of JBH1. Table 4.2 reveals the identities among the putative OYEs found in JBH1 and those known to be able to denitrate NG. Although identities among putative OYE1, OYE2, OYE3, OYE4, and OYEs known to denitrate NG are low, they are similar to the identities observed among OYEs known to transform NG, such as XenA, XenB, Onr, NerA, and YqjM, ranging between 48% and 29% identity. Conserved regions present in all flavoproteins known to denitrate NG are also present in OYE1, OYE2, OYE3, and OYE4.

**Table 4.2. Identities of the sequence alignments of OYEs identified in JBH1 and other OYEs known to denitrate NG\***

	OYE1	OYE2	OYE3	OYE4	XenA	XenB	NerA	Onr	YqjM
OYE1	-	30	32	31	31	44	46	47	46
OYE2	30	-	49	34	43	38	38	32	41
OYE3	32	49	-	36	41	36	33	35	36
OYE4	31	34	36	-	45	36	37	37	30
XenA	31	43	41	45	-	35	32	30	40
XenB	44	38	36	36	35	-	46	48	32
NerA	46	38	33	37	32	46	-	42	37
Onr	47	32	35	37	30	48	42	-	29
YqjM	46	41	36	30	40	32	37	29	-

\*Amino acid % identity. XenA from *Pseudomonas putida* (accession number AF154061.1), XenB from *Pseudomonas fluorescens* (accession number AF154062.1), Onr from *Enterobacter cloacae* (accession number YP\_003612825), NerA from *Agrobacterium radiobacter* (accession number YP\_002540225), and YqjM from *Bacillus subtilis* (accession number NC\_000964.3).

To investigate if any of the putative OYEs identified in the JBH1 genome was responsible of the initial transformation of NG to 1 MNG, *OYE1*, *OYE2*, *OYE3*, and *OYE4*, were cloned and overexpressed in Rosetta 2 *E. coli* cells. A control containing the unaltered pET24a vector was used for comparison. Results from these experiments



indicated that *E. coli* cells (control) are highly efficient in the denitration of NG to DNG (both isomers). However, *E. coli* cells do not produce notable amounts of 1 MNG, which is the major denitration intermediate observed in JBH1 cell extracts in the presence of NADPH. Only the Rosetta 2 cells containing pJBH1-3 produced significant ( $p < 0.001$ ) amounts of 1 MNG (Figure 4.2).

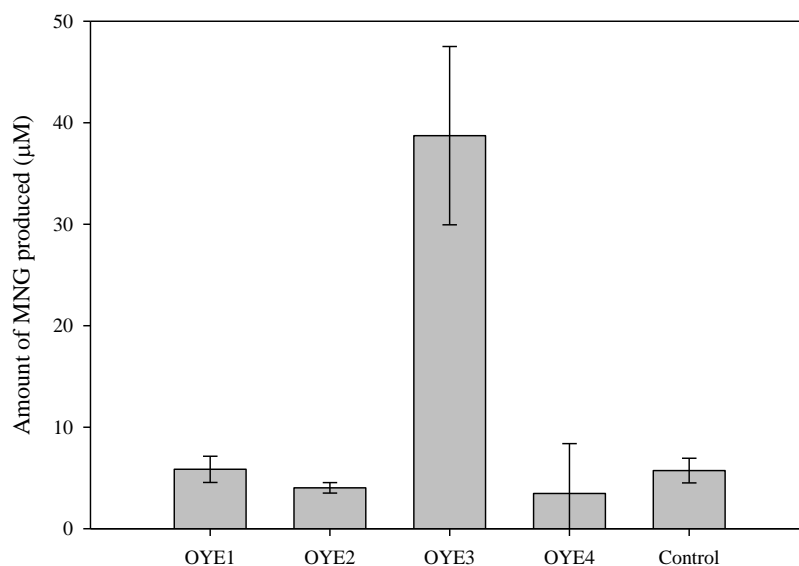


Figure 4.2. Amount of 1 MNG produced in *E. coli* clones containing overexpressed OYE1, OYE2, OYE3, OYE4, and the control

#### 4.4.2 TRANSFORMATION OF MNG

To investigate the mechanism involved in the transformation of 1 MNG in JBH1, glycine buffer (100 mM, pH 8.3) with 1 MNG (70 µM) was amended with JBH1 cell extracts. No MNG disappearance was observed during the first 10 minutes of incubation. After 10 minutes, the mixture was amended with ATP and magnesium and the disappearance of MNG was observed (Figure 4.1).

Further experiments conducted using cell extracts from JBH1 (glycine buffer, 100 mM) to degrade 1 MNG (50  $\mu$ M) indicated that the degradation rate can be increased 3 times by increasing the concentration of ATP from 100  $\mu$ M to 200  $\mu$ M, and that increasing the initial MNG concentration 3 fold (150  $\mu$ M MNG, with 300  $\mu$ M ATP) increases the degradation rate 5 times. In the absence of additional  $MgCl_2$ , the degradation rate is reduced by 40%, indicating this reaction is magnesium dependent, as observed in glycerol kinases. ADP is highly inhibitory to the reaction; in the presence of 100  $\mu$ M of ATP, the addition of 100  $\mu$ M ADP completely inhibited the MNG transformation reaction (Table 4.3). This observation explains the rapid drop in 1 MNG concentration in JBH1 cell extracts observed during the first few minutes after the addition of ATP (Figure 4.1), followed by a fast drop in degradation rate, as ADP accumulates from the transformation of ATP. Nitrite and nitrate were measured in experiments conducted with JBH1 cell extracts after the transformation of 1 MNG in the presence of Mg-ATP. No nitrite or nitrate was detected in any of the samples.

**Table 4.3 Transformation of 1 MNG in JBH1 cell extracts under different experimental conditions**

Experimental condition	Rate ( $\mu$ mol/mg protein h)
MNG 50 $\mu$ M, ATP 100 $\mu$ M, pH 7.6	0.49 $\pm$ 0.01
MNG 50 $\mu$ M, ATP 100 $\mu$ M, pH 8.3	0.51 $\pm$ 0.02
MNG 50 $\mu$ M, ATP 100 $\mu$ M, pH 9.3	0.90 $\pm$ 0.17
MNG 50 $\mu$ M, ATP 200 $\mu$ M, pH 8.3	1.59 $\pm$ 0.18
MNG 50 $\mu$ M, ATP 300 $\mu$ M, pH 8.3	0.74 $\pm$ 0.02
MNG 100 $\mu$ M, ATP 200 $\mu$ M, pH 8.3	1.55 $\pm$ 0.17
MNG 150 $\mu$ M, ATP 300 $\mu$ M, pH 8.3	2.68 $\pm$ 0.03
DEAD enzyme	-0.02 $\pm$ 0.02
MNG 50 $\mu$ M, ATP 100 $\mu$ M + 100 $\mu$ M ADP	-0.18 $\pm$ 0.14

To identify the gene(s) involved in the transformation of MNG a fosmid library was created. A single *E. coli* clone (from 300 screened) containing the plasmid pJBH1A3 was capable of transforming MNG, which was verified by its capability of releasing nitrite from 1 MNG transformation. A culture containing pJBH1A3 (resting cells, OD ~ 0.5) was capable of removing 40  $\mu$ M MNG in 4 hrs. None of the other 299 clones were capable of releasing nitrite after incubation with MNG over night. pJBH1A3 was purified and sequenced. This fosmid is 42 kb, from which 8 kb constitute the vector pCC1FOS.

Transposon mutagenesis of pJBH1A3 was used to identify the specific genes involved in MNG transformation. Several clones lost the ability to transform 1 MNG after transposon mutagenesis. Sequencing results from these clones indicated that there are two genes involved in the transformation of MNG.

One of the genes identified after transposon mutagenesis was most similar to a glycerol kinase (GlpK) with 67% identity to a glycerol kinase from *Cellulomonas* sp. To obtain additional evidence of the involvement of this glycerol kinase homolog in the transformation of 1 MNG in JBH1, the putative kinase from JBH1 was cloned into *E. coli*. The gene was overexpressed and experiments conducted using resting cells indicated that 1 MNG could be transformed in *E. coli* clones containing the cloned gene from JBH1, and could not be transformed in control cells containing pET24a without the gene. Transformation was verified by the formation of nitrite in cultures exposed to MNG overnight.

Cell extracts from overexpressed putative kinase clones (100  $\mu$ M glycine buffer, 100  $\mu$ M ATP, 50  $\mu$ M 1MNG) were used to investigate the effect of pH on the activity of the enzyme (Figure 4.3) and this activity was compared with the activity observed in

JBH1 cell extracts and cell extracts from *E. coli* control (transformed with pET24a vector without insert). Similar glycerol kinases, such as that from *E. coli* and *Cellulomonas* sp. have optimum pH values higher than 9.3 (39, 79). At pH 7.6 the 1MNG transformation rate in JBH1 cell extracts was approximately 50% lower than that observed in cell extracts containing the overexpressed kinase, and consistent with rates expected based on observations of NG biodegradation in JBH1 whole cells. There was no significant difference between transformation rates between pH 7.6 or 8.3 in either JBH1 cell extracts or cell extracts with the overexpressed kinase. At both pH values the transformation rate in the controls was close to zero. At pH 9.3 there was a notable increase in transformation rate in all three treatments (including the control). As the degradation rate also increased in the control, it is possible that MNG hydrolysis might be partly responsible for the disappearance of MNG at this higher pH. Nonetheless, at this point we have not quantified how much of the increase in degradation is a result of hydrolysis and the optimum pH of the enzyme was not identified.

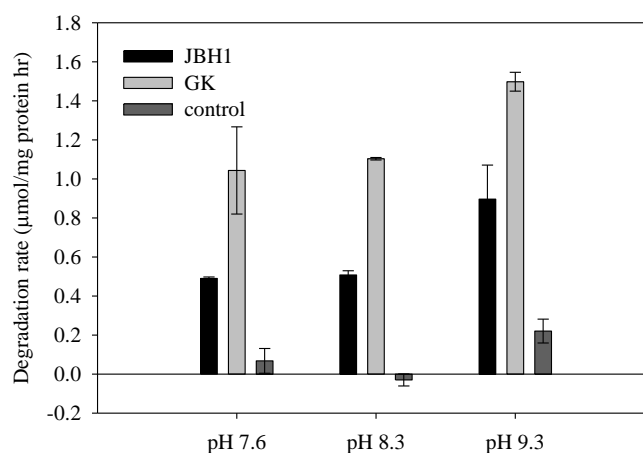


Figure 4.3. 1 MNG transformation rate in cell extracts from JBH1, *E. coli* cells with overexpressed glycerol kinase from JBH1, and *E. coli* control

The second gene identified by transposon mutagenesis to be involved in MNG transformation is a gene composed of 252 amino acids that contains a FIC (filamentation induced by cAMP) domain. The FIC motif is defined by a conserved amino acid sequence HPF<sub>x</sub>(D/E)GN(G/K)R, and it has been found in most forms of life from bacteria to humans (94). It was recently reported that some FIC-domain proteins are capable of using ATP to catalyze the addition of adenosine monophosphate (AMP) to other proteins (94) in a process known as AMPylation. AMPylation is a posttranslational modification mechanism similar to phosphorylation, in which the added moiety changes the activity of the modified protein (44, 47). Although effort was made to overexpress the protein containing the FIC motif in *E.coli*, the experiment was unsuccessful (no band could be observed using protein gel electrophoresis, even after using various incubation conditions).

Sequence analysis of the fosmid segment containing the two enzymes indicated that the two enzymes are about 3.3 kb apart (Figure 4.4) and that there is a potential hairpin structure located in the region following the enzyme containing the FIC motif which could act as an intrinsic translation terminator (93), suggesting that both genes of interest could be located on different operons. To our knowledge there are no similar gene arrangements in the Genbank database.

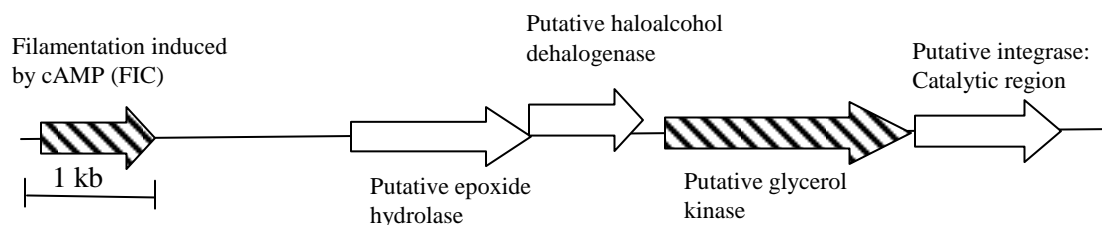


Figure 4.4. Annotation of pJBH1A3 (region of interest)

## 4.5 DISCUSSION

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The fact that the formation of 1,2 DNG and 1 MNG in JBH1 cell extracts was stoichiometric to the disappearance of NG after the addition of NADPH (the total nitroester concentration remained constant) indicated that enzymatic processes requiring NADPH can remove the first and second nitro groups from NG and that the third nitro group cannot be removed by the same denitration mechanism. These observations are consistent with previous results obtained with purified OYEs (13), as no known OYE is capable of removing all three nitro groups from NG. The subsequent disappearance of MNG in cell extracts following the addition of ATP-Mg indicated that the mechanism responsible for the transformation of 1 MNG is ATP dependent and not NADPH dependent and that an enzymatic mechanism different from that previously known to denitrate NG (which requires NADPH) is responsible for the transformation of the intermediate.

A second indication that an OYE is involved in the transformation of NG in this strain was obtained after overexpressing several OYE homologues identified in the genome of JBH1 in *E. coli* cells. Although *E. coli* cells are highly efficient in the denitration of NG to DNG (to form both isomers), the fact that the cells are not capable of producing notable amounts of 1 MNG (which is the major denitration intermediate observed in JBH1 cell extracts in the presence of NADPH), indicated that OYE3 is likely the enzyme involved in the selective transformation of DNG to 1 MNG in JBH1, since only the Rosetta 2 cells containing pJBH1-3 produced significant amounts of 1 MNG. However, at this point we do not have an understanding of how NG is initially denitrated

to DNG and further analysis using the purified enzymes are required to differentiate NG denitration to DNG by *E.coli* from that catalyzed by the overexpressed enzymes.

The identification of one of the genes as a glycerol kinase homolog is consistent with our results that indicate that the transformation of 1 MNG in JBH1 cell extracts is ATP dependent. Glycerol kinases have been widely studied in the past (5, 16, 41, 63, 64, 95, 96) and are known to transfer a phosphate group from ATP to glycerol forming glycerol-3 phosphate. The glycerol-3 phosphate is then oxidized to dihydroxyacetone phosphate and enters glycolysis/gluconeogenesis to be used as a carbon source (63, 64). Several C3 substituted glycerol analogs can also be transformed by glycerol kinases (17, 23). Substituents that can be tolerated include 3-chloro-1,2 propanediol, 3-fluoro-1,2 propanediol, 3-butene-1,2-diol, 1,2,4-butanetriol, and 3-mercaptopropane-1,2-diol, although to our knowledge there are no reports of transformation of nitroester compounds by a similar enzyme. Glycerol kinases are less tolerant towards C2- substituted propanediols (17, 23), which could explain findings in a previous report that indicate that JBH1 cannot grow on 2 MNG (42), which is a C2 substituted propanediol. This finding also highlights the significance of OYE3 in JBH1, which selectively produces the 1 MNG isomer; if the 2MNG isomer was produced instead, growth through the pathway catalyzed by a glycerol kinase would not be possible.

Based on the known mechanisms used by glycerol kinases to transform substituted glycerols, we proposed two different transformation pathways for 1 MNG (Figure 4.5). In the first pathway, 1MNG is phosphorylated at the carbon opposite to the nitroester group. Here, no nitrite or nitrate is released after the glycerol kinase catalyzes the reaction in the presence of the Mg-ATP complex. Then, the phosphorylated nitroester

would be introduced into a central metabolic pathway, such as glycolysis, in which the final nitrite group is released. In the second proposed pathway, phosphorylation would occur at the carbon that contains the nitroester bond, displacing the nitro group and releasing nitrate. Then the phosphoglycerol would be further degraded through glycolysis or a similar pathway to release CO<sub>2</sub>.

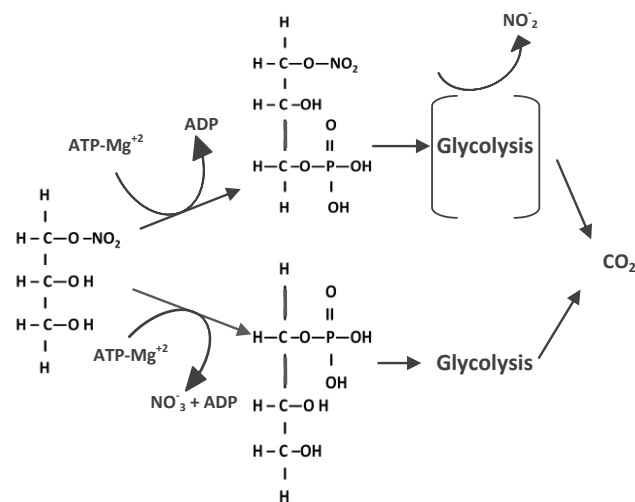


Figure 4.5. Proposed transformation pathways of 1 MNG catalyzed by the glycerol kinase in JBH1

Since no nitrite or nitrate was detected in any of the samples, even after a transformation of 60 μM 1MNG (in the case of the second hypothesis, this reaction would yield 60 μM nitrate, which is about 10 times higher than the nitrate detection limit of the analytical method used) results suggest that phosphorylation is occurring at the carbon opposite to the one containing the nitroester bond, producing 1-nitro-3-phosphoglycerol. The formation of a molecule with a molecular weight of 217 (consistent with that of 1-nitro-3-phosphoglycerol) was verified by LC/MS. However, because of the limited amounts of materials, we have not yet verified the identity of this intermediate by other techniques, such as NMR. Since nitrite was observed in resting cell experiments



using *E. coli* cells with the overexpressed glycerol kinase from JBH1 (and not in the *E. coli* control), it is believed that *E. coli* contains the appropriate enzymatic pathway to further transform 1-nitro-3-phosphoglycerol, releasing nitrite.

Despite the high identity between the 1 MNG kinase from JBH1 and glycerol kinases (*Cellulomonas* sp 67 %; *E. coli* 52%), there are significant differences among them at the glycerol binding site where glutamine (E84 in *E. coli*, E82 *Cellulomonas* sp.) is substituted for an alanine (ionizable acidic to non polar) and tyrosine (Y135 in *E. coli*, Y133 in *Cellulomonas* sp.) is substituted by valine (non ionizable polar to non polar) in the kinase from JBH1. It is possible that smaller terminals of Ala and Val (relative to tyrosine and glutamine) could allow more room for the binding of 1 MNG to the enzyme although it is still unknown how MNG binds to the enzyme.

Regulation of the glycerol kinase is complex and has been widely studied (16, 41, 63, 64, 96). Glycerol kinase activity is regulated by the sugar phosphotransferase system (PTS) in firmicutes and enterobacteriaceae. PTS is a complex system that involves carbohydrate transport and phosphorylation system and uses phosphoenolpyruvate (PEP) as the phosphoryl donor. In certain gram positive bacteria the glycerol kinase is phosphorylated on a histidyl (His232 in *Enterococcus*) residue by a histidine-containing phosphocarrier protein (HPr), which greatly increases the activity of the kinase (95). In *E. coli*, regulation of the glycerol kinase is achieved through allosteric inhibition due to complex formation with enzyme III<sup>Glc</sup> (41), which is also phosphorylated by HPr.

In addition to differences between 1 MNG kinase in JBH1 and glycerol kinases at the glycerol binding site, differences at the regulatory site are also evident. For instance, kinase in JBH1 lacks the histidyl residue that is commonly phosphorylated in glycerol

kinases from gram positives to activate the enzyme (95). Likewise, the region known to bind to III<sup>Glc</sup> in *E. coli* is also not present in the kinase from JBH1. These observations could indicate that a different regulatory mechanism from the two presented above might be involved in the regulation of GlpK in JBH1.

The facts that no nitrite production was observed in any of the cultures containing fosmids where the enzyme encoding the FIC conserved region was mutated (following transposon mutagenesis), suggests that the enzyme may be involved in the regulation of the 1 MNG kinase. This transformation would be analogous to that observed in the activation of glycerol kinases of gram positive strains, where before phosphorylation activity is significantly lower than that observed after the posttranslational regulatory transformation. However, since 1 MNG kinase was overexpressed to conduct enzyme activity tests, the activity was still greater than in the wild type, even in the absence of the enzyme containing the FIC motif. Nonetheless, at this time, we do not have a clear understanding on how the enzyme containing the FIC motif is involved in the transformation of MNG and further research should be conducted to investigate this aspect.

The presence of an OYE that selectively produces the 1 MNG isomer, a kinase capable of transforming 1 MNG, and a protein containing a FIC motif which possibly regulates the activity of the glycerol kinase, in a single microbe, gives JBH1 the ability to incorporate MNG into a productive degradation pathway, allowing it to grow on NG as the sole source of carbon and nitrogen.

## CHAPTER 5: BIODEGRADATION OF NITROGLYCERIN IN POROUS MEDIA

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### 5.1 ABSTRACT

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Nitroglycerin (NG) is a toxic explosive found in soil and groundwater at old manufacturing plants and military sites. When it enters the subsurface, NG behaves as a dense non aqueous phase liquid (DNAPL) and mobilizes rapidly due to its relatively high solubility in water. Through column experiments this work evaluates the biodegradation of NG and NG degradation metabolites in porous media under various conditions. Results indicate that after inoculation with *Arthrobacter* sp. strain JBH1, nitroglycerin can be biologically mineralized in porous environments, even in the absence of additional sources of carbon and nitrogen, and that nitrite and dinitroglycerin can be used as indicators of biological degradation. The presence of organic carbon in the soil can result in NG sorption to the soil matrix, possibly reducing the concentration of bioavailable NG, which could result in recalcitrance. The presence of common co-contaminants, trinitrotoluene and dinitrotoluene can also potentially result in reduced degradation rates. However, low pH (5.1) and high NG concentrations (1.22 mM) do not greatly affect degradation rates by JBH1.

### 5.2 INTRODUCTION

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Nitroglycerin is a liquid at standard temperature and pressure with solubility values ranging between 1.27 and 2 g/L at ( between 15 and 25°C) (48) and a freezing temperature of 13°C (31) . NG has been widely used for the production of dynamite (31, 86), propellants (55, 86), and smokeless powder (86). Because of improper handling,

release, and disposal to the environment, NG has been found in contaminated soil and groundwater at various locations (29, 51, 75) and exposure to NG can be harmful both to wildlife and humans (10).

NG (sp. gravity =1.59 at 25°C (31)) behaves as a dense non-aqueous phase liquid (DNALP) when it enters the subsurface environment migrating downward into the aquifer and remaining trapped within the pores or accumulating on impervious layers as lenses or pools (90). Since nitroglycerin is a powerful impact sensitive explosive, drilling or excavating at a contaminated site to remove or simply determine the size, shape, and location of the source zone is extremely dangerous. Therefore, understanding the biodegradation of NG in porous media and using the appropriate biodegradation indicators could provide researchers and engineering professionals the means to quantify NG degradation *in situ* and possibly conduct inverse-modeling predictions without exposing themselves to an explosion hazard.

Biological denitration of NG has been studied in some detail, mostly focusing on NG behavior in wastewater treatment systems (2, 10, 25, 89). As a nitrogen source, NG is denitrated sequentially to 1,2 or 1,3-dinitroglycerin (DNG) followed by 1 or 2-mononitroglycerin (MNG) under both aerobic and anaerobic conditions, resulting in release of nitrite (10, 25, 51, 53, 89). NG can be biologically denitrated (completely or partially) by bacteria isolated from contaminated soil and sediments (51, 53, 89) when supplied a source of carbon and energy. Recently, a strain (*Arthrobacter* sp. strain JBH1)(42), was isolated and confirmed to grow on NG as the sole source of carbon and nitrogen (completely mineralizing nitroglycerin and NG degradation intermediates).

The objective of this study was to evaluate the ability of JBH1 to degrade NG in porous media in the absence of other sources of organic carbon or nitrogen.

Biodegradation indicators were identified through a series of column experiments, which were also aimed at quantifying the extent and rate of biological degradation using low sorption sand and high organic content NG contaminated soil. The potential for using *Arthrobacter* JBH1 for bioaugmentation in soils in which NG degradation is slow or incomplete was examined, and the effects of pH, NG concentration, and the presence of common co-contaminants dinitrotoluene (DNT) and trinitrotoluene (TNT) on NG biodegradation by this strain were evaluated.

### 5.3 MATERIALS AND METHODS

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*Chemicals.* NG, 1,2 DNG, and 1,3 DNG standard solutions in acetonitrile were purchased from Cerilliant (Round Rock, TX). Methanol Chromasolv plus for HPLC > 99.9% (Sigma Aldrich, Montana) was used for chromatography; glycerol used for NG synthesis was spectrophotometric grade > 99.5% and was purchased from Acros Organics. 2,4-Dinitrotoluene (97%) and 2,6-Dinitrotoluene (98%) were obtained from Aldrich (Milwaukee, WI), and 2,4,6-Trinitrotoluene (30% water) was obtained from Chem Service Inc, (West Chester, PA). TNT, 2,4 DNT and 2,6 DNT stock solutions were produced by adding excess TNT or DNT to DI water and allowing it to dissolve for a week; stock was filtered through a 0.2  $\mu\text{m}$  filter prior to performing any experiments. NG was synthesized using sulfuric acid, nitric acid, and glycerol with a proportion of 3.3 to 2.7 to 0.3 (V/V/V), respectively (86) .

*Analytical.* Nitroglycerin, 1,2DNG, 1,3DNG, 1 MNG, 2 MNG, TNT, 2,4 and 2,6 DNT were measured using an Agilent HPLC equipped with a Supelguard guard column

(10 x 4.6 mm, Supelco, Bellefonte, PA), a Supelco LC-18 column (250 x 4.6 mm, 5  $\mu$ m, Supelco) and a UV detector. Methanol-water (50% v/v or 40 % v/v) was used as the mobile phase with a flow rate of 1ml/min and the wavelength was set at 214 nm. Nitrite was analyzed as previously described (42). Total protein was quantified using the Micro BCA Protein Assay Kit (Pierce Biotechnology, IL). Total organic carbon in soil samples was analyzed using a Shimadzu TOC analyzer.

*Culture Media and Buffer Solutions.* Mineral medium (pH 7.2) consisted of (in grams per liter)  $\text{Na}_2\text{HPO}_4$ , 2.66;  $\text{KH}_2\text{PO}_4$ , 1;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.02;  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.01;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.003; and 1.0 ml of trace elements solution per liter. The trace elements solution was prepared as previously described (83).

*Soils.* Uncontaminated ASTM C-33 concrete sand (U.S. Silica) was sieved through a 2 mm and a 1 mm mesh. The fraction between 1-2 mm was washed with hydrogen peroxide (10%) for 20 minutes, rinsed with DI water, autoclaved and placed in the oven to dry at 105°C for 24 hours. The NG contaminated soil sample that was previously used for the isolation of the NG degrader *Arthrobacter* JBH1(42) was used for the second column experiment and was obtained in Kenil, NJ. A second NG contaminated sample consisted of river sediment from the Potomac River, WV. The two NG contaminated samples and the cleaned and sterilized sand were analyzed for sorption as follows: NG dissolved in water (868 mg/L) was placed into 10 mL glass vials and sodium azide was added (200 mg/L) to inhibit biological growth. Soils were weighed and placed in the vials containing NG in water. Vials were shaken for 48 hours and aqueous samples were then analyzed for NG concentration. Samples of soil were centrifuged using a Millipore Ultrafree-MC centrifuge filter to remove excess water. 60  $\mu$ g of each

centrifuged sample was collected and placed in a clean centrifuge filter and 200  $\mu$ L of methanol were added. Each tube was vortexed and incubated for 10 minutes at room temperature. The sample was then centrifuged and the leachate was collected and analyzed for NG. The properties of the soils used are listed in table 5.1.

**Table 5.1. Soil Characteristics**

<b>Parameter</b>	<b>Sand</b>	<b>Kenvil, NJ</b>	<b>Potomac River, WV</b>
Fraction of organic carbon, $f_{oc}$	<0.0003	0.09	0.03
Partitioning coefficient for NG, $K_d$	$\sim 0$	2.93	0.8
% Fines (passing 75 $\mu$ m sieve)	-	46	15
Soil bulk density ( $\text{g}/\text{cm}^3$ )	1.56	1.14	1.40
Initial NG conc. (mg/kg)	-	50	160

*Bacterial Culture.* *Arthrobacter* JBH1 initially isolated from Kenvil, NJ (42) was maintained in mineral media supplemented with NG (0.3 mM).

*Column Experiments.* Two sterile Omnifit glass columns (10 mm ID x 250 mm L) were filled half way with mineral medium and then sand was slowly added into the column and compacted by vortexing to avoid air pocket formation. The columns were inoculated using 1 mL of *Arthrobacter* JBH1(42) (OD 600  $\sim$ 0.3) before securing the top plate. Glass columns were placed horizontally with one open end and the other end was connected to a 100 mL air tight syringe (Hamilton 1100) attached to a syringe pump. Syringes were sterilized using hydrogen peroxide (10%) for 10 minutes (3 X). NG solution in mineral medium (1.35 mM) was injected at an initial flow rate of 0.15 ml/hr. Microbial culture was allowed to grow inside the sand columns before any measurements were taken. Once the microbial matrix was established and biodegradation was observed (constant nitrite concentration from the column's effluent was observed), the flow rate was lowered to 0.075 ml/hr. A collection vial was placed on ice at the effluent of the columns and effluent was collected for 2-3 hours (approximately 200  $\mu$ L of sample were

collected from each column). The samples were later diluted in ultra pure water to a total volume of 3000  $\mu$ L and were analyzed for NG, NG degradation intermediates, and nitrite concentration. After the conclusion of the experiment, sand was carefully pushed out of the columns, cut into ~2 cm segments, and analyzed for protein content.

For the second experiment the two columns were packed with soil from Kenil, NJ (as described above) and inoculated using 1 mL of JBH1(42) (OD 600 ~0.3). NG solution in mineral medium (0.5 mM) was injected into the soil filled columns at 0.15 mL/hr for 17 days. The third column experiment was conducted using sediment from the Potomac River, WV, (as described above). Here only one of the columns was inoculated with 1 mL of JBH1(42) (OD 600 ~0.3). NG solution in mineral medium (0.43 mM) was injected into both columns at 0.15 mL/hr.

*Effects of pH and NG concentration on biological degradation.* Serum vials containing mineral medium (20 mL) and NG stock solution (1 mL) were inoculated with JBH1. pH in the vials was adjusted using 1 N HCl. The pH values investigated were 5.1, 5.7, 6.2, 6.5 and 7.2. No higher pH conditions were investigated due to observations from previous abiotic experiments that indicated that NG hydrolyzes at pH of 7.5 or greater (Appendix B), complicating the analysis of results. An abiotic control was used at a pH of 7.2. Subsequently, serum vials containing mineral medium (20 mL) were inoculated with JBH1 at pH 7.2. NG concentration in vials was adjusted by adding different volumes of NG solution to each vial. NG concentrations studied were 0.26, 0.33, 0.82, 1.04, and 1.22 mM.

*DNT, TNT and NG mixtures.* TNT, 2,4 DNT, and 2,6 DNT stock solutions were added to serum vials (in duplicate) containing NG in mineral media (100 mg/L) as



follows: NG, 2,4 DNT (30 mg/L), 2,6 DNT (20 mg/L), and TNT (14 mg/L) abiotic control, NG positive control, 2,4 DNT (55 mg/L, 40 mg/L, and 20 mg/L), 2,6 DNT (35 mg/L, 25 mg/L, and 15 mg/L, TNT), TNT (21 mg/L, 14 mg/L, and 8 mg/L). All vials but the abiotic control were inoculated with 1 mL of *Arthrobacter* JBH1 (OD600 ~ 0.3) and were maintained on the shaker for 48 hours, at which point NG and nitroaromatic concentrations were measured.

## 5.4 RESULTS AND DISCUSSION

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### 5.4.1 COLUMN EXPERIMENTS

Low sorptivity sand ( $K_d$  for NG $\approx$ 0) with a negligible amount of organic carbon was used to simplify initial column experiments aimed at evaluating the ability of JBH1 to degrade NG in a porous medium. There was a decrease of 21% (from 0.95 mM to 0.75 mM) in effluent NG concentration as a result of decreasing water velocity in the column from 0.43 to 0.21 cm/hr (increase in hydraulic retention time (HRT) from 53 to 101 hours) (Figure 5.1). Because little sorption to the sand is expected and abiotic reactions are unlikely at this pH (based on preliminary studies conducted to determine the extent of hydrolysis in 48 hours as a function of pH), these results show evidence that biological degradation can be important in sand in the absence of additional sources of carbon. No transformation of NG was observed in shake flask experiments containing sterile sand, further indicating that the disappearance observed in columns was of biological origin. 1,2 DNG was observed at the effluent at concentrations between 0.13 and 0.21 mM throughout the experiment indicating some incomplete degradation. High concentrations of nitrite (0.73 mM at high HRT and 1.1 mM at low HRT) were also observed, indicating

approximately 75-95 % of the nitrogen contained in the NG that was degraded was recovered at the columns' effluents in the form of DNG or nitrite.

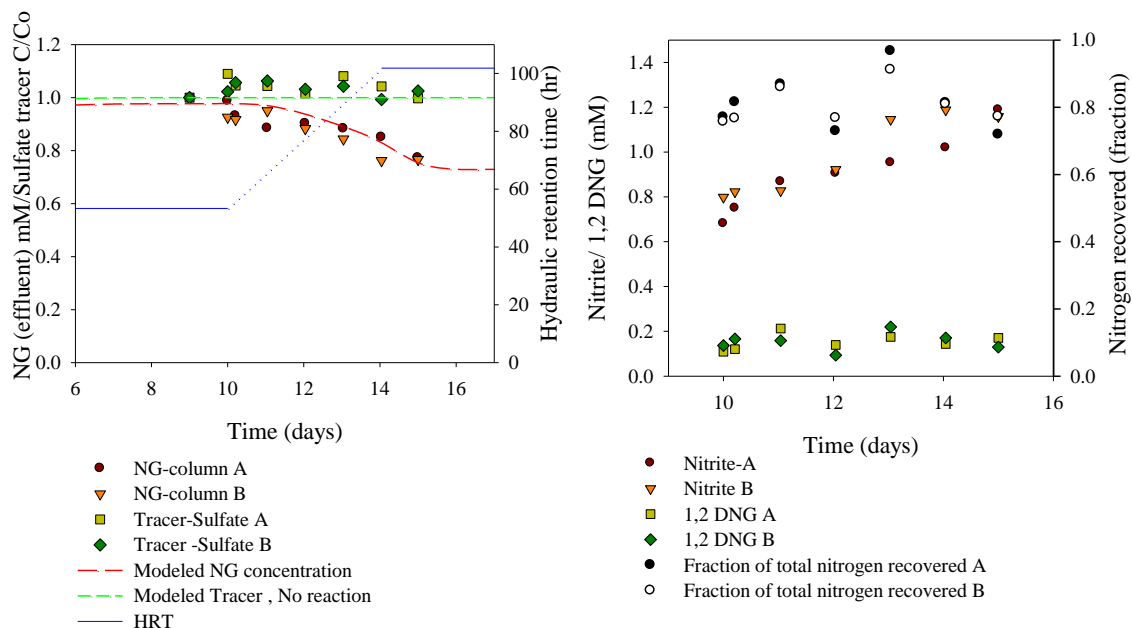
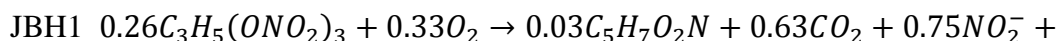


Figure 5.1. Transient change in NG effluent concentration as a result of a change in water velocity compared to a tracer (left). Nitrite, 1,2DNG and fraction of nitrogen recovered in the form of 1,2 DNG and nitrite (right)

After the completion of the experiments, sand was extracted from the columns and protein measurements in sand showed protein concentrations of up to 27 mg/kg sand in the first 5 cm of column near the entrance. Concentration then decreased to 15 mg/kg sand, remaining constant throughout the rest of the column (data not shown). This concentration of protein through the column suggests that NG degradation is taking place throughout the column and that possibly higher availability of oxygen and other nutrients at the columns entrance results in higher growth rates.

Based on the stoichiometric equation that describes NG aerobic degradation by



$0.75H^+ + 0.17H_2O$  (42), 1 mol of NG requires 1.27 mols of  $O_2$ . The degradation observed in this system (1.35 – 0.75 mM, with 0.15 mM 1,2 DNG, due to incomplete denitration), would require 0.57 mM of oxygen, which greatly exceeds the solubility concentration of oxygen in water (oxygen in the influent is close to saturation values but no additional oxygen is provided to the columns). As a result, it is expected that all dissolved oxygen in the columns is depleted. However, at this point we do not have an understanding of degradation mechanisms under different electron acceptor conditions, although there is a report of anoxic growth in the presence of nitrite in other *Arthrobacter* strains which are closely related to JBH1(30) and JBH1 contains genes that encode proteins involved in nitrite ammonification such as the nitrite reductase [NAD(P)H] large subunit (EC 1.7.1.4), NirB, and the nitrite reductase [NAD(P)H] small subunit (EC 1.7.1.4), NirD.

Assuming degradation kinetics are first order with respect to NG concentration, the one-dimensional transport of the dissolved NG plume in porous media with linear sorption and reaction can be described by the general advection-dispersion equation as

$$\frac{\partial C}{\partial t} \left( 1 + K_d \frac{\rho_b}{n} \right) = -v \frac{\partial C}{\partial x} + D_L \frac{\partial^2 C}{\partial x^2} - kC \quad (66),$$

where  $C$  is the concentration of NG,  $\rho_b$  is the bulk density of the soil,  $n$  is the porosity,  $v$  is the velocity,  $D_L$  is the hydrodynamic dispersion coefficient, and  $k$  is a first order degradation rate constant. The term  $1 + K_d \frac{\rho_b}{n}$  is known as the retardation factor  $R$ , which represents the apparent reduction in advection velocity of the pollutant due to sorption processes and  $v_s$  (apparent advection velocity of the pollutant) =  $v/R$ .

As mentioned earlier, the reduction in flow rate after 10 days of operation resulted in a decrease in NG concentration at the effluent. The decrease in concentration as a function of time was modeled using the unsteady state analytical solution for the equation presented above (66), which describes the concentration as a function of time at a given column length (L) and a changing water velocity,  $C(L, t) = \frac{C_0}{2} \exp\left(\frac{-kL}{v_s}\right) \operatorname{erfc}\left(\frac{L-v_st}{2\sqrt{\alpha_L v_st}}\right)$ . The velocity,  $v_s$ , in this case is  $v$  ( $R=1$ , with no sorption), was estimated based on the systems flow rate, the sectional area of the column, and the porosity. The empirical formula  $\alpha_L=0.1L$  (66), was used to calculate the proportionality constant  $\alpha_L$  and  $k$  was identified as 0.14/d (using first order decay kinetics (68)) (Figure 5.1). This rate constant (0.14/d) is in the same order of magnitude to those observed in column experiments with toluene (0.65/d), o-xylene (0.0625-0.262/d), p-xylene and m-xylene (0.35/d), and benzene (0.5/d) (6). This is an interesting finding as to our knowledge, the degradation of NG in soil had not been quantified and there was no clear understanding of how much degradation could be expected in a porous system and the potential for using bioremediation as a treatment mechanism.

In the presence of sorption, the apparent retention time of NG in the soil is higher. Assuming that natural organic matter dominates the sorption mechanism of NG in soils, the soil-water partitioning coefficient can be expressed as  $K_d=K_{oc}f_{oc}$ , where  $K_{oc} = \frac{C_{oc}}{C_w}$ , and  $f_{oc}$ ,  $C_{oc}$ , and  $C_w$  represent the weight fraction of the solid that represents organic matter, the concentration of compound associated with natural organic matter, and the concentration in the aqueous phase, respectively. The  $K_{oc}$  of NG was calculated using EPI Suite (85) as 130.8.

The NG-contaminated soil sample obtained from Kenvil, NJ had higher organic content ( $f_{oc} \approx 0.09$ ) than other contaminated soil samples, and therefore was selected to study the effect of sorption on the degradation of NG in a porous medium.  $K_d$  for this soil was measured as 2.93. A mixed culture capable of growing on NG and producing both DNG isomers was initially present in soil from the site.

No NG was detected in effluents from columns packed with soil from Kenvil and injected with NG (0.5 mM) (Figure 5.2). However, both DNG isomers were detected at relatively high concentrations. This observation can be explained by the much lower  $K_{oc}$  of DNG, which is only 3.95 (85), two orders of magnitude lower than NG. Therefore, after NG is denitrated to DNG, the less sorptive DNG isomers can migrate more rapidly, having a higher apparent velocity, and lower retention time, resulting in lower degradation. The breakthrough concentration as a function of time without degradation was predicted using the equation presented above. After 17 days of operation, the expected effluent concentration (no reaction) would be 0.31 mM. The summation of the observed nitroester species in the effluent was 0.19 mM, indicating some of the NG (0.31-0.19 mM) was completely degraded in the system. Nitrite recovery was low, consistent with incomplete denitration of NG, which can be caused by low bioavailability due to sorption. No significant amounts of MNG were detected in the effluent at any time. Results indicate that DNG isomers could be used as biotransformation indicators as part of a monitored natural attenuation approach or to quantify NG degradation during bioremediation.

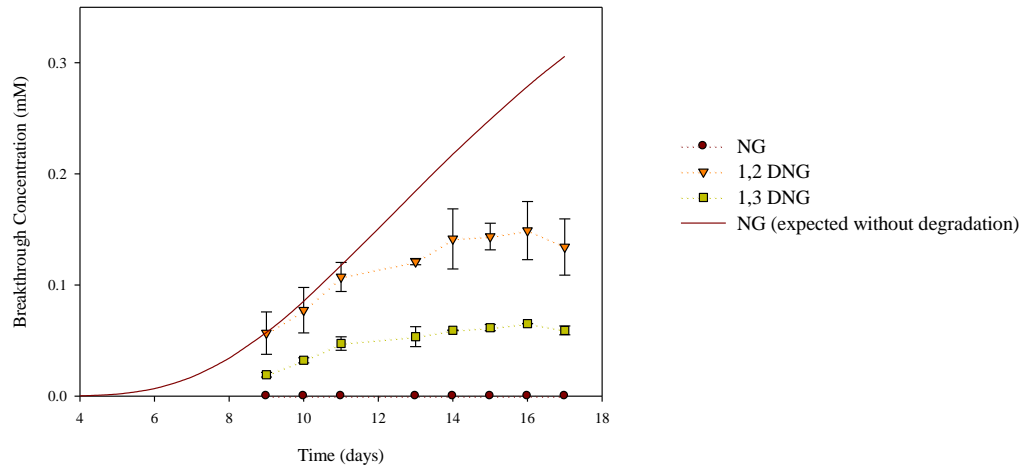


Figure 5.2. Nitroester concentration in column effluent resulting from degradation in sorptive soil. Data represent the average obtained from two columns and bars represent the range

Incomplete denitration of NG has been observed in the past with simultaneous accumulation of either DNG or MNG (13, 51, 89) . Incomplete NG denitration resulting in the accumulation of 1,2 DNG was observed in an NG-contaminated sediment sample. In a third set of experiments, both columns were packed with sediment. To investigate the potential for using JBH1 for bioaugmentation purposes, one of the columns was inoculated with JBH1. NG degradation occurred in both systems. Nonetheless, after steady state conditions were achieved, NG concentration in the effluent of the column that only contained the native microbial community was about 0.17 mM (39% of the influent concentration), whereas NG concentration in the column that was inoculated with JBH1 was only 0.01 mM (2 % of influent concentration). Additionally, as expected, 1,2 DNG accumulation in the column that had only the native population was 0.13 mM ( 31 % of influent nitroester) compared to that obtained in the presence of JBH1, which was only 0.02 mM ( 6% of influent nitroester concentration) (Figure 5.3). These results show that bioaugmentation with JBH1 could potentially reduce NG concentrations at a

contaminated site with slow or incomplete degradation. Results were modeled using the equation presented earlier. The reaction rate observed in the column that was inoculated was 0.58/d, and was only 0.12/d in the column that contained only the native microbial community. The degradation rate 0.58/d is 4 times higher than that observed in sand columns. It is possible that the native microbial community positively contributed to the transformation of NG, so that 1,2 DNG could be further degraded by JBH1, therefore increasing the degradation rate. Additionally, it is possible that the higher concentration of NG in the sand columns (1.35 mM as compared to 0.42 mM in the columns packed with sediments) resulted in a decreased degradation rate. The effect of NG concentration on degradation rate and the effect of some site specific characteristics that could affect the degradation of NG by JBH1 are described below.

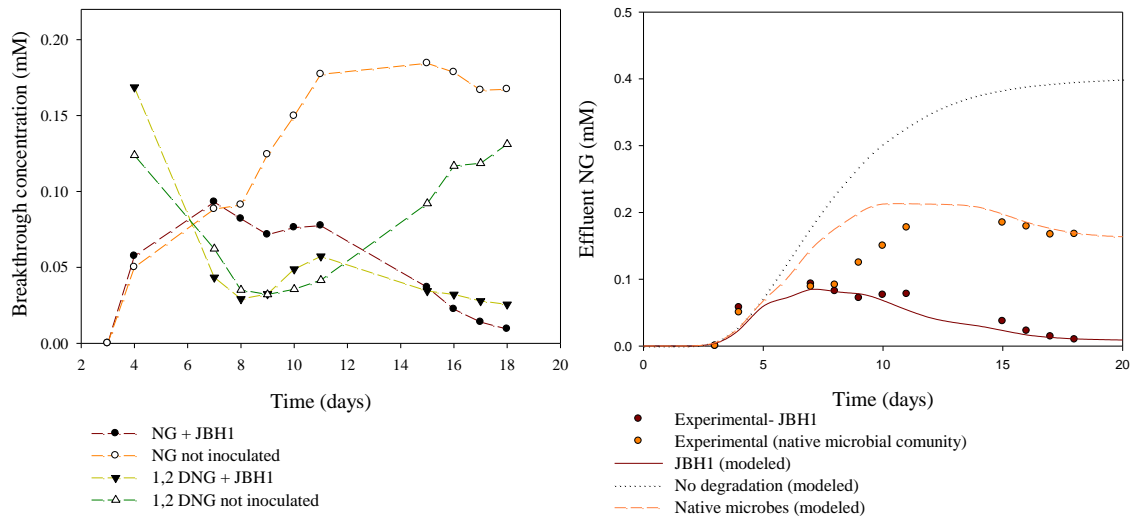


Figure 5.3. NG degradation in Potomac River sediment. Left: Comparison of degradation by native microbial community and inoculation with JBH1. Right: Modeling results

#### 5.4.2 SHAKE FLASK EXPERIMENTS

Low pH conditions may result during NG biodegradation (nitrite production) in poorly buffered soils. To investigate how pH could affect NG degradation rates, flasks containing NG at different pH values were inoculated with JBH1. At pH values close to neutral (7.2), the observed initial degradation rate was approximately twice as high as the rate observed at a pH of 5.1 (Figure 5.4). pH higher than 7.2 was not investigated to avoid hydrolytic degradation.

High NG concentrations ( $> 0.5$  mM) also resulted in a decrease in degradation rates (42). At low NG concentrations (0.33 mM), the initial degradation rate was 2.3  $\mu\text{mol NG/mg protein hr}$  and was reduced to just 1.3  $\mu\text{mol NG/ mg protein hr}$  at 1.22 mM (Figure 5.4). Since 1.22 mM is well below the solubility concentration of NG (6.6 mM) it is possible that NG concentrations in groundwater may exceed the inhibitory concentration. However, other mechanisms such as dispersion and sorption could rapidly reduce the aqueous concentration to values where degradation is rapid.

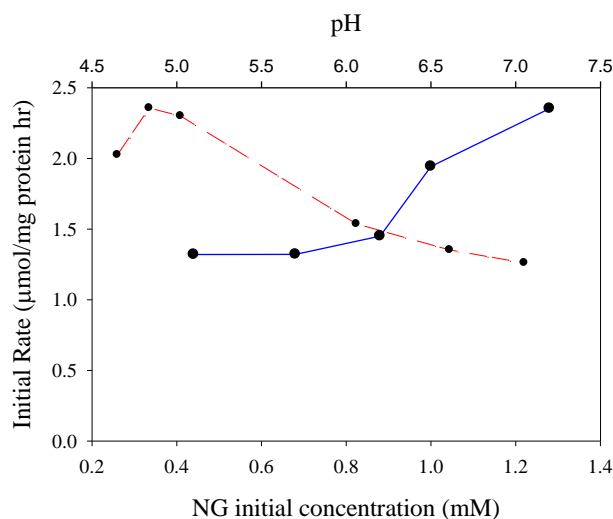


Figure 5.4. Effect of pH (blue line) and NG concentration (red line) on NG degradation rate



The simultaneous or sequential utilization and disposal of several explosives and propellants at military sites in conjunction with their concurrent manufacture at production plants has resulted in the presence of other explosives besides NG at contaminated sites (75, 88). These other explosives include TNT, which has been the most widely used military explosive since World War I (86) and DNT, which is not manufactured as a main explosive, but is used as an energetic binder in some propellant and high explosive formulations (55), and may also serve as an antifreeze (31). The solubilities of TNT and DNT in water are low (2,4DNT ~200mg/L(55), TNT ~100mg/L (70)), making them recalcitrant and due to their toxicity they are expected to be a major concern at explosives contaminated sites. Numerous reports have focused on the biodegradation of TNT and DNT in mixed cultures, pure cultures and even *in situ* (33, 34, 83, 99) and inhibition of 2,4DNT and TNT on 2,6DNT biodegradation has been previously observed (37), which raises the question of whether any of these co-contaminants is inhibitory to NG biodegradation.

Shake flasks containing NG and 2,4DNT, NG and 2,6 DNT, or NG and TNT were inoculated with *Arthrobacter* JBH1. Flasks containing 2,6 DNT showed no difference from the biological control that contained only NG, indicating 2,6 DNT is not inhibitory to NG degradation by JBH1(Figure 5.5). The presence of 2,4 DNT reduced the degradation rate as compared to the NG biological control indicating that 2,4 DNT is inhibitory to JBH1 during NG degradation, and inhibition increased as the concentration of DNT increased. No degradation of 2,4 or 2,6 DNT by JBH1 was observed during this experiment. The presence of TNT completely inhibited the degradation of NG by JBH1 even at concentrations as low as 7 mg/L, with over 90% of the initial NG remaining after

168 hours. TNT was slightly degraded in flasks containing 7mg/L TNT inoculated with JBH1, although a progressive loss of activity was observed, suggesting the possibility that a toxic intermediate was accumulating. This observation is consistent with previous observations of enzymatic inhibition during TNT reduction where it is believed that a nitrosodinitrotoluene intermediate reacted with the enzyme resulting in enzyme inactivation (67).

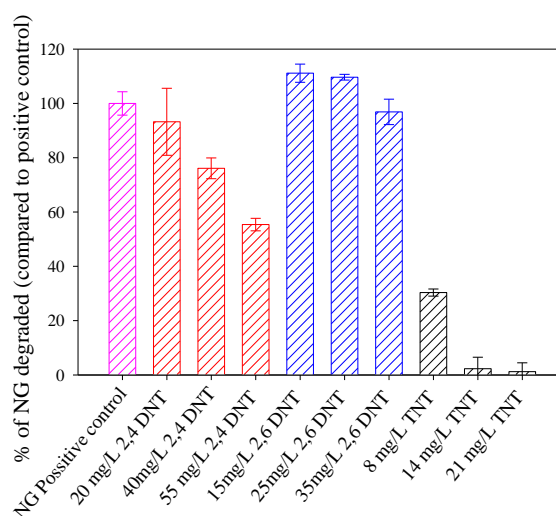


Figure 5.5. NG degradation after 48 hours after inoculation with JBH1 in the presence of common explosive co-contaminants

Results presented here show the ability of JBH1 to mineralize NG in a porous system in the absence of other sources of carbon and nitrogen. Nitrite and dinitroglycerin were identified as indicators of biodegradation, which could be used in the field as part of a monitored natural attenuation approach. In the presence of microbes capable of incomplete mineralization, inoculation of an NG contaminated soil matrix with JBH1 can result in a reduced NG concentration, indicating JBH1 could potentially be used as part

of a bioaugmentation strategy in the field, although further studies are required to evaluate the adaptability of this strain to the field.

NG degradation rates could vary from site to site, and even after bioaugmentation with JBH1 lower rates could be observed at sites with high NG concentration and low pH. The presence of organic carbon in the soil could result in NG sorption to the soil matrix, potentially reducing the concentration of bioavailable NG, which could lead to NG persistence. Additionally, the presence of TNT and 2,4 DNT can potentially reduce the rates of NG biodegradation. However, the degradation rates presented here establish an initial understanding of the extent of NG biodegradation that could be observed in a contaminated field, which was unknown.

## CHAPTER 6: CONCLUSIONS AND ENGINEERING SIGNIFICANCE

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Contamination of soil and groundwater with nitroglycerin is of great concern not only due to its explosive nature but due to its high toxicity. Excavating and drilling for contaminated soil removal or even plume identification can be very dangerous and therefore the bioremediation and natural attenuation of NG at sites contaminated with explosives is attractive. Although previous research had been conducted to study the microbial transformation of NG as a source of nitrogen in aqueous solutions, there was no evidence that NG biodegradation is possible in soil and groundwater, where the presence of other sources of carbon and energy is not expected and low pH, nutrient limitation, and the presence of other common explosives may occur. Understanding the biological degradation of nitroglycerin under these conditions provides the foundation for understanding natural attenuation of NG and for the development of treatment strategies that involve bioremediation at sites contaminated with explosives.

This work presents evidence that nitroglycerin can serve as a sole source of carbon and nitrogen and that complete mineralization by a pure culture (*Arthrobacter* sp. strain JBH1) is possible. The fact that NG can be the sole growth substrate makes bioremediation and natural attenuation more sustainable alternatives, as no additional sources of carbon are required to achieve complete detoxification from NG contamination.

This work also shows evidence that in *Arthrobacter* JBH1 the degradation pathway is highly specific and includes the formation of the intermediates 1,2 DNG and 1 MNG, with simultaneous release of nitrite. 1 MNG is then converted to central metabolic intermediates, which can be used as a source of carbon and energy, releasing carbon dioxide and forming biomass.

Flavoproteins, members of the Old Yellow Enzyme (OYE) family have been previously associated with the removal of the first and second nitro groups from NG resulting in the formation of MNG. Results from this investigation indicated that a putative OYE from JBH1 is responsible for the selective production of the 1 MNG isomer in the presence of NADPH. The mechanism and the enzymes responsible for the transformation of MNG were previously unknown and this investigation has identified two enzymes involved in the pathway. These enzymes include a MNG kinase homolog which is responsible for phosphorylating 1 MNG at the C3 position, producing an intermediate compound which could be further metabolized to carbon dioxide and nitrite. The second enzyme was identified as a member of the Fic family which might be involved in the regulation of the MNG kinase, although more research is needed to investigate the exact mechanism.

Results from this investigation indicate that NG can be biologically degraded in porous media, and that biological degradation can play an important role in the fate of nitroglycerin in the subsurface. Therefore bioremediation and monitored natural attenuation can now be considered as potential treatment mechanisms. Additionally, this work identified biodegradation indicators (1,2 DNG, nitrite) that can be used to monitor NG biodegradation away from the source, reducing the potential for explosion near

DNAPL plumes. Potential bottlenecks for NG *in situ* biodegradation and some of the aspects that need to be taken into account when observing recalcitrance *in situ* are also described in this document.

The presence of a microbial community capable of mineralizing NG is fundamental for complete detoxification of a contaminated site and this work identified a pure culture that may be a candidate for bioaugmentation in the absence of appropriate communities. JBH1 is capable of growing on NG without any additional sources of carbon and can degrade NG at low pH values found at contaminated sites. Additionally, this work shows that JBH1 can grow on NG at relatively high NG concentrations which may be observed near source zones, and can be grown on rich medium (such as LB) without losing the ability of transforming NG, making it an interesting candidate for bioaugmentation. However, inhibition of NG biodegradation by TNT was significant, and therefore *Arthrobacter* JBH1 is not recommended for bioaugmentation at NG contaminated sites which also contain TNT.

This work represents the foundation for understanding bioremediation and natural attenuation of NG at contaminated sites and will help engineers and scientist design appropriate treatment strategies that include bioremediation and natural attenuation for soils and aquifers contaminated with NG and to understand the mechanisms controlling the transformation of NG *in situ*. However, several questions still remain and should be considered as part of future work.

In terms of the degradation pathway, additional lines of evidence are required that verify that the compound produced after phosphorylation by MNG kinase is the 1-nitro-3-phosphoglycerol. Additionally, at this point it is unknown how the phosphorylated

intermediate is transformed within the cell to release nitrite, and how the enzyme containing the Fic motif is involved in the transformation of 1 MNG. There is no clear understanding of how NG is transformed to 1,2 DNG or the specific enzyme that catalyzes this reaction. Identifying the enzymes and the regulatory mechanisms associated to these enzymes could potentially allow us to identify ways to improve the rates of degradation (changing regulation, selecting for better enzymes). Additionally, understanding how this pathway evolved would give us a more complete understanding on how widespread this mechanism is, and of the likelihood of finding similar pathways in other strains.

Up until now, all bioaugmentation tests have been conducted using laboratory scale settings and we do not have a clear understanding on how this microbe will behave under site specific conditions. Therefore, conducting pilot scale experiments would give us a better understanding of the specific conditions that could affect NG degradation by this microbe, and will help us understand and predict the extent of biodegradation that could be expected at a contaminated site bioaugmented with *Arthrobacter* JBH1.

## APPENDIX A: NG TRANSPORT AND DEFINITION OF NATURAL ATTENUATION

---

When NG enters an aquifer it behaves as a dense non-aqueous phase liquid (DNAPL). When minor spills occur, NG migrates into the soil, mostly sorbing onto natural occurring organic carbon and later desorbing into clean water passing through, therefore arriving as a dissolved contaminant to the water table (90). In contrast, after a large spill, NG can migrate through the vadose zone and penetrate the water table fingering downward into the saturated zone. Here NG first forms a continuous body, that migrates through the subsurface invading soil pores and media fractures. Once the source of NG stops, the continuous body continues to migrate due to gravity and forms discontinuous blobs that remain in pores, as residual non-aqueous liquid (Figure A.1).

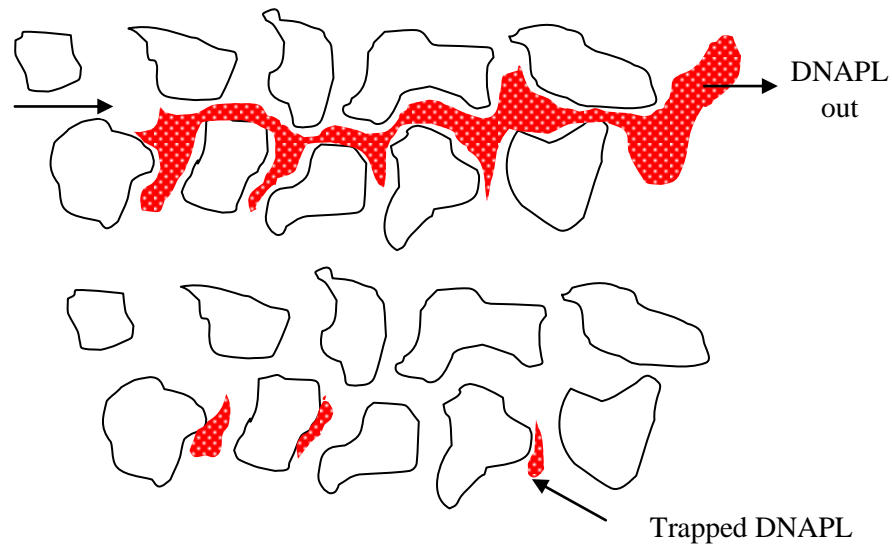


Figure A-1. Representation of DNAPL spill in porous medium



As the density of DNAPLs is higher than that of water, DNAPLs tend to migrate to the bottom of the aquifer or until it reached an impervious layer, creating DNAPL pools (90). The dissolution of DNAPL into groundwater is a complex phenomenon. Studies of dissolution of residual DNAPL using saturated soil columns showed initial effluent concentrations near solubility values, which later dropped to almost non-detectable, as most of the DNAPL mass was removed. Additionally, these studies identified aqueous phase flow rate, NAPL saturation, and porous media grain size as the critical parameters that influence mass transfer (43, 56, 65).

Natural attenuation can be described as the observed reduction in contaminant concentration as the contaminant migrates from the source into the environment (90). How far the contaminant will migrate and how long it will persist in the environment depends primarily on fate and transport processes which include dissolution, dispersion, advection, sorption, and other biotic and abiotic processes, which in the case of nitroglycerin include biological and hydrolytic degradation.

## A-1 ADVECTION

---

Advection is a primary mechanism involved in the movement of dissolved NG in an aquifer. This movement is caused by the bulk groundwater movement, and is highly dependent on aquifer properties such as hydraulic conductivity and effective porosity (90). The one dimensional change in concentration as a function of time resulting from advection can be calculated as

$$\frac{\partial C}{\partial t} = -v_x \frac{\partial C}{\partial x} \quad (\text{A.1})$$

Where  $C$  is the contaminant concentration,  $t$  is time, and  $v_x$  is the linear velocity in the direction  $x$  (66).

## A-2 HYDRODYNAMIC DISPERSION

---

As water flows through porous media it encounters obstacles and blockages that cause channeling, by-passing, or confinement (66). Here some water molecules move faster and some slower than the average groundwater velocity, and if water is carrying NG, it appears to be mixed together. This mechanism is known as mechanical dispersion and cannot be separated from effective molecular diffusion in pore waters resulting in a combined hydrodynamic dispersion coefficient. Based on field observations, mechanical dispersion coefficients can be modeled as a function of the linear groundwater velocity.

The hydrodynamic dispersion coefficient can be described as

$$D_L = \alpha_L v + D_m \quad (\text{A.2})$$

Where  $D_L$  is the longitudinal hydrodynamic dispersion coefficient,  $\alpha_L$  is a proportionality constant,  $v$  is the velocity, and  $D_m$  is the molecular diffusion coefficient. Molecular diffusion in groundwater typically occurs very slow compared with mechanical dispersion, and therefore the longitudinal hydrodynamic coefficient is commonly approximated by (66)

$$D_L = \alpha_L v \quad (\text{A.3})$$

An empirical approximation is commonly used to predict the proportionality constant,  $\alpha_L = 0.1x$ , where  $x$  is the distance traveled (66). The one dimensional change in

concentration as a function of time due to advection and hydrodynamic dispersion can be described by

$$\frac{\partial C}{\partial t} = -v_x \frac{\partial C}{\partial x} + D_L \frac{\partial^2 C}{\partial x^2} \quad (\text{A.4})$$

### A-3 SORPTION OF NG TO SOIL MEDIA

---

Sorption is the process through which chemicals become associated with solid phases. During adsorption, the molecules are attached to a two-dimensional surface; during absorption, molecules penetrate into a three-dimensional matrix (71). Sorption processes may play a crucial role in the fate of NG in contaminated soil and groundwater as identical NG molecules will behave differently if they are surrounded by water molecules or sticking to the surface of a solid. Additionally, dissolved NG is more accessible to microorganisms and macroorganisms than sorbed NG.

There are many models that can be used to describe partitioning of a contaminant between solid and aqueous phases, including the Langmuir and the Freundlich Isotherms (71). At low concentrations, the sorption behavior can be linear and can be expressed as described by the solid-water partitioning coefficient,  $K_d$ , which relates the contaminant concentration in the solid phase ( $C_s$ ) with the concentration in the aqueous phase ( $C_w$ )(71):

$$K_d = \frac{C_s}{C_w} \quad (\text{A.4})$$

$K_d$  is a function of many parameters which include the concentration of compound associated with natural organic matter  $C_{oc}$ , and the weight fraction of solid

that represents natural organic matter  $f_{oc}$ . However, the concentration of compound associated with the mineral surface and the specific surface area of the relevant solid may also play very important roles in the determination of this parameter (71). It can be assumed that natural organic matter dominates the sorption of NG in organic rich soils. A relationship for the solid-water distribution coefficient as a function of  $C_{oc}$ , and  $f_{oc}$  can be described as

$$K_d = K_{oc} \cdot f_{oc} \quad (A.5)$$

$$\text{Where } K_{oc} = \frac{C_{oc}}{C_w} \quad (A.6)$$

Log  $K_{oc}$ , has been previously measured experimentally (2.77) (55, 82), and is relatively low, and therefore NG is not highly sorptive. NG partitioning coefficients between 0 and 7.3 L/kg (20, 61) have been obtained experimentally with different soil samples of varying organic carbon contents.

Sorption tends to reduce the apparent solute transport velocity and it removes some of the contaminants from groundwater via sorption to the aquifer matrix (90). If we define  $S$  as the sorbed concentration, the loss of contaminant to the aquifer material during mass transfer in an aquifer can be expressed as

$$\frac{\partial C}{\partial t} = -v_x \frac{\partial C}{\partial x} + D_L \frac{\partial^2 C}{\partial x^2} - \frac{\rho_b}{n} \frac{\partial S}{\partial t} \quad (A.7)$$

Where  $\rho_b$  is the bulk density of the aquifer matrix and  $n$  is the porosity. Equation A.4 can be transformed to  $\frac{\partial S}{\partial t} = K_d \frac{\partial C}{\partial t}$  and substituted into equation 3.7, yielding

$$\frac{\partial C}{\partial t} = -v_x \frac{\partial C}{\partial x} + D_L \frac{\partial^2 C}{\partial x^2} - K_d \frac{\rho_b}{n} \frac{\partial C}{\partial t} \quad (A.8)$$

Equation 3.8 can be rearranged to yield

$$\frac{\partial C}{\partial t} \left( 1 + K_d \frac{\rho_b}{n} \right) = -v_x \frac{\partial C}{\partial x} + D_L \frac{\partial^2 C}{\partial x^2} \quad (\text{A.9})$$

Which describes the one-dimensional transport of a contaminant in porous medium (66) with no reaction.

Volatilization, abiotic and biological degradation can also greatly affect the fate of contaminants in aquifers. To simplify the solution of the system, degradation is often assumed to be first order with respect to the substrate concentration. Here equation A.9 can be transformed to

$$\frac{\partial C}{\partial t} \left( 1 + K_d \frac{\rho_b}{n} \right) = -v_x \frac{\partial C}{\partial x} + D_L \frac{\partial^2 C}{\partial x^2} - kC \quad (\text{A.10})$$

Where  $k$  is a first order degradation rate. However, modeling studies have found that using first order kinetics in microbial degradation studies may not fully describe the conditions of the system, as they do not take into consideration aspect such as lag phase and microbial growth (18, 90). Nonetheless, equation A.10 is widely used to model the fate of single contaminants in the subsurface (66).

## APPENDIX B: HYDROLYSIS OF NITROGLYCERIN IN AQUEOUS SOLUTION AS A FUNCTION OF PH

---

The abiotic degradation of NG in the subsurface is mostly controlled by hydrolysis. A reaction in which a water molecule substitutes for another group or atom is called hydrolysis (72). The product resulting from the hydrolytic degradation of a compound by water is usually of less environmental concern than the parent compound due to its different properties. It has been previously observed that nitroglycerin can be hydrolyzed through three different mechanisms which include nucleophilic substitution, elimination of beta-hydrogen, and elimination of alpha hydrogen (15). It was previously determined that rate of NG hydrolysis at high pH (12) can be described by a second order rate equation as

$$\frac{d[NG]}{dt} = -k[NG][OH^-] \quad (B.1)$$

With k values ranging between  $1.24 \times 10^{-3}$  (10°C) and  $2.36 \times 10^{-2}$  (25°C) (15), yielding both nitrite and nitrate, with the formation of formate and other unidentified volatile organics. Later experiments conducted at lower pH (9) and higher temperatures (50°) also identified 2-hydroxypropanedial and glycolic acid as hydrolytic intermediates (36).

Previous chapters describe how NG can be degraded by microbes, and how biodegradation affects the fate of NG in porous media. To properly design the experimental setups and determine experimental conditions, it was necessary to conduct some preliminary experiments. The hydrolytic transformation of NG as a function of pH

was investigated to properly select the experimental pH range to be used in biological experiments such that hydrolytic degradation is not significant.

## B-1 MATERIALS AND ANALYTICAL METHODS

---

*Chemicals.* NG standard solution in acetonitrile was purchased from Cerilliant (Round Rock, TX). Methanol Chromasolv plus for HPLC > 99.9% (Sigma Aldrich, Montana) was used for chromatography; glycerol used for NG manufacturing was spectrophotometric grade > 99.5% and was purchased from Acros Organics.

*Nitroglycerin Synthesis.* NG was synthesized using sulfuric acid, nitric acid, and glycerol with a proportion of 3.3 to 2.7 to 0.3 (V/V/V), respectively (86) . Components were mixed in a constant temperature chamber at 25°C for 60 minutes and the resulting NG NAPL was then transferred to filtered DI water using a pipette. This process was repeated until the pH was stable and low nitrate concentration was detected in the aqueous phase. Following, the NG was pipetted into a sterilized water bottle and was allowed to dissolve in a shaker for at least three days, to produce a dissolved NG stock solution.

*Analytical.* Nitroglycerin was measured using Agilent HPLC equipped with a Supelguard guard column (10 x 4.6 mm, Supelco, Bellefonte, PA), a Supelco LC-18 column (250 x 4.6 mm, 5  $\mu$ m, Supelco) and a UV detector. Methanol-water (50% v/v) was used as the mobile phase with a flow rate of 1ml/min and the wavelength was set at 214 nm. Total organic carbon in soil samples was analyzed using a Shimadzu TOC analyzer following manufacturer's instructions.

*Buffer Solution.* Phosphate buffer was prepared with  $\text{Na}_2\text{HPO}_4$  (2.66 g/L) and  $\text{KH}_2\text{PO}_4$  (1 g/L).

*Hydrolysis Experiments.* Triplicate 20 mL serum bottles were filled with 10 mL of phosphate buffer, 5 mL of NG in water (735mg/L), and NaOH 1 N was utilized to reach desired pH values. pH values utilized for this experiment were 4.8, 6.5, 7.5, 8.5, 9.5, and 11. Serum bottles were covered from the light and mixed continuously; samples were taken at time 4, 28, and 48 hours and put into 2mL HPLC vials. HCl (1N) was used to lower pH to 4 in all samples collected and the vials were maintained in the refrigerator (4°C) until HPLC analysis the following day. HPLC analysis was conducted to measure NG concentration in all samples, and IC was used to determine nitrate concentration in some of the samples.

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## B-2 RESULTS AND DISCUSSION

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No accumulation of DNG or MNG was observed at any of the pH values investigated. NG hydrolysis at initial pH 11 was very successful (>61% NG decrease and high nitrate formation), but because the initial solution was poorly buffered, high nitric acid production lowered the pH to 8 after 48 hours (figure B-1), stopping the hydrolytic process in the samples. No degradation was observed at pH values of 4.8 and 6.5 (evident by no decrease in NG concentration and no evidence of nitrate formation), and slight hydrolysis was observed at pH 7.5 (decrease in NG concentration < 5%, very little nitrate formation).



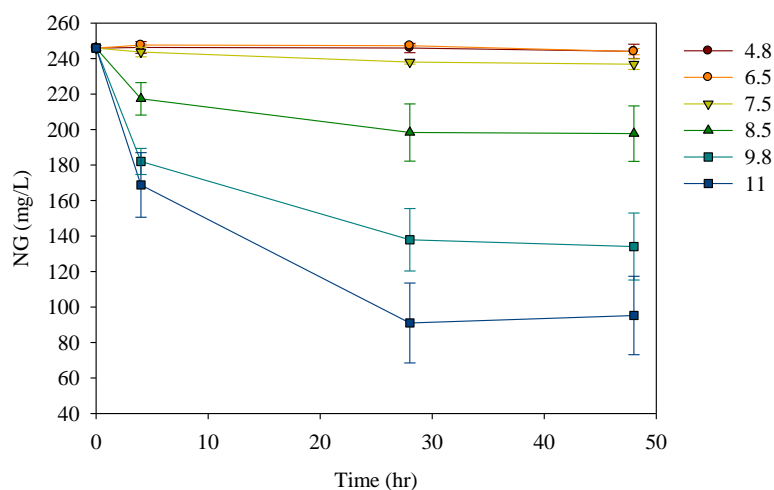


Figure B-1. Hydrolytic degradation of NG as a function of time at various pH values. (data represent average  $\pm$  standard of triplicates)

Solving equation B.1 we obtain  $\ln\left(\frac{C}{C_0}\right) = -k[OH^-]t$ . At a constant time (48

hours) the relationship between  $\ln(C/C_0)$  can be plotted as a function of pH (Figure B-2).

The linear relationship between pH and  $\ln(C/C_0)$  is evident at high pH values (higher than 7.4), indicating that NG can be rapidly hydrolyzed at high pH and that degradation rate increases as a function of pH.

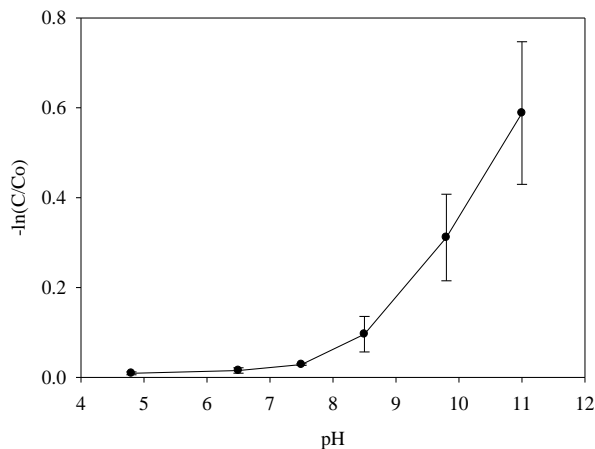


Figure B-2. NG relative concentrations as a function of pH after 48 hours. Data represent the average  $\pm$  the range

A prior study conducted by Capellos et al (1984) (15) used a second order kinetics rate model of the form

$$\ln \frac{[NG]}{[OH^-]} = \ln \frac{[NG]_o}{[OH^-]_o} + ([NG]_o - [OH^-]_o)kt \quad (B-2)$$

to describe the hydrolytic degradation of NG at pH 12. Using this approach, the reaction rate  $k$ , at pH 11 was identified as  $2.03 \times 10^{-3} \text{ M}^{-1}\text{s}^{-1}$  which is consistent with values observed by Capellos et al. in 1984. pH change in time was estimated based on a mass balance approach with the electron neutrality equation of the system, assuming all hydrolytic degradation resulted in the formation of 3 moles of nitrate per mol of NG.

It is proposed that alkaline treatment could be an appropriate mechanism to treat NG contaminated soils. However, more research should be conducted to test this hypothesis. Based on results obtained here, experiments aimed at studying biological degradation described in chapters 3, 4 and 5, were conducted at pH values below 7.5 to ensure that the observed degradation was biological.

## APPENDIX C: SOIL CHARACTERIZATION AND SORPTION OF NG TO SOIL MEDIUM

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As presented in appendix A, NG is known to sorb onto the soil particles and therefore it is necessary to determine the partitioning coefficients of NG to the soil samples used in experiments in chapter 5. Doing so allowed for the proper selection of soils to investigate different aspects of NG mobilization in a porous medium. Preliminary experiments are described in the remainder of this section.

### C-1 SOIL CHARACTERIZATION

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Different soils were used for sorption experiments. Soil 1 consisted of uncontaminated ASTM C-33 concrete sand (U.S. Silica) which was sieved through a 2 mm. Collected material was then sieved through a 1 mm sieve mesh to remove fines. Fraction between 1-2mm was then washed with hydrogen peroxide (10%) for 20 minutes, rinsed with DI water, autoclaved and placed in the oven to dry at 105°C for 24 hours. The second sample was NG contaminated soil obtained in Kenil, NJ (50 mg NG/kg soil). The third sample consisted of contaminated sediment from the Potomac River (160 mg NG/kg soil), and the last sample was NG contaminated soil from Barksdale, WI. Soil samples from Kenil, Barksdale, and sediment from the Potomac River were characterized based on size distribution. Here, sieves number 6, 10, 40, 70, 100, and 200 and were used and were placed on a sieve shaker for 10 minutes.

NG dissolved in water (868 mg/L) was placed into 10 mL glass vials and sodium azide was added (200 mg/L) to inhibit biological growth. Different amounts from all soils were weighted and placed in the vials containing NG in water. Vials were set on a shaker for 48 hours and aqueous samples were then analyzed for NG concentration. Samples of

soil were centrifuged using a Millipore Ultrafree-MC centrifuge filter to remove excess water. 60µg of each centrifuged sample was collected and placed in a clean centrifuge filter and 200 µL of methanol were added. Each tube was vortexed and incubated for 10 minutes at room temperature. The sample was then centrifuged and the leachate was collected. The process was repeated and leachate was analyzed for NG.

## C-2 RESULTS AND DISCUSSION

Based on the Unified Soil Classification System (USCS), all soil samples can be classified as sandy soils, with less than 50% passing sieve #200 (75 µm) and more than 12% fines (7) (Figure C-1).

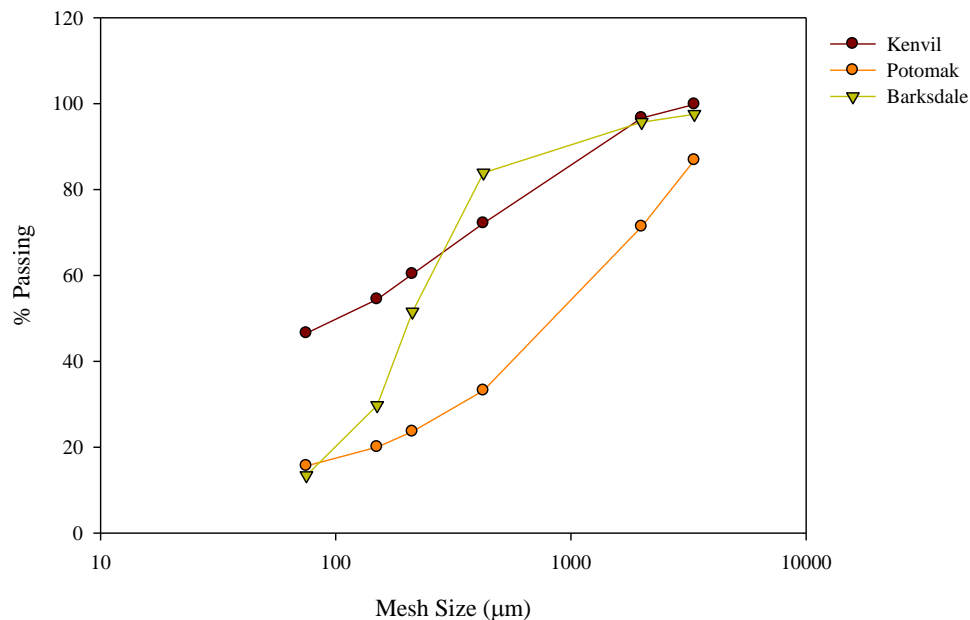


Figure C-1. Size distribution analysis

The fraction of organic carbon in each sample was calculated using a Shimadzu TOC analyzer following manufacturer's instructions. Samples from the Potomac River and

from Kenvil, NJ showed a linear relationship between aqueous concentration and soil concentration with slopes ( $K_d$ ) of 0.8 and 2.93, respectively. Sand showed very low sorption (NG concentrations in the aqueous phase did not change after 48 hours equilibration time) and the sample from Barksdale showed some sorption, although no relationship between aqueous and solid concentrations could be established from the data obtained. These relationships between aqueous and soil concentrations can be explained from the relationship between  $K_d$  and  $f_{oc}$  presented in equation B.5, which indicates that if sorption is dominated by organic matter,  $K_d$  increases as a function of the fraction of organic carbon contained in the sample. Based on the  $K_d$  values obtained for Kenvil soil and Potomac sediment, and the fraction of organic carbon measured (Table C-1), assuming sorption is dominated by organic matter in the soil, the  $K_{oc}$  can be calculated as  $29 \pm 4.7$ .

**Table C. 1. Fraction of organic carbon in soil**

Sample	Fraction of Organic carbon	$K_d$ for NG (L/Kg)
Sand	0.0	~0
Potomac	3.0	0.8
Barksdale	0.5	*
Kenvil	8.8	2.93

\* Did not present a linear relationship at the concentrations investigated

Based on the observations presented in this section, it is recommended that experiments aimed at studying NG biodegradation in porous medium without sorption (to simplify the system) can be conducted with the sand sample described above. Experiments that intend to include sorption into the system can use the soil from Kenvil, NJ, which is the most sorptive soil from all samples investigated.

## APPENDIX D: MIXED CULTURE CAPABLE OF GROWING ON NG AS THE SOLE SOURCE OF CARBON AND NITROGEN

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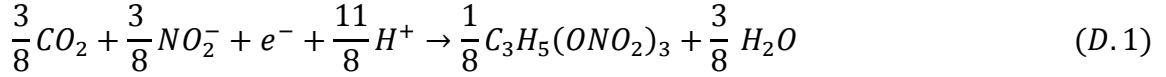
Previous studies conducted to investigate biodegradation of NG were conducted using alternative sources of carbon. During wastewater treatment additional sources of carbon are commonly present and therefore no carbon addition is required. During bioremediation, however, the soil may be lacking alternative sources of carbon for NG degrading microbes, and adding such chemicals is usually costly and can be dangerous. Therefore, determining if microbial cultures are capable of degrading NG as the sole source of carbon, nitrogen, and energy, is essential if bioremediation of NG contaminated sites is possible.

### D-1 THEORETICAL CONSIDERATIONS

---

To date very little is known about the microbial transformation of NG as a sole source of carbon and nitrogen. Many reports indicate NG can be transformed in the presence of other sources of carbon, indicating that it is not yielding net energy to the transforming cell, and therefore requires other substrates as a source of energy (77). Smetes and coworkers conducted a thermodynamic analysis of the NG degradation reaction and concluded that the oxidation of glycerol to carbon dioxide yields enough energy to allow microbes to completely denitrate NG and still have energy available for growth (77). Here we present a more extensive thermodynamic analysis following the procedure proposed by Rittmann and McCarty (69), aiming to determine the viability of using NG

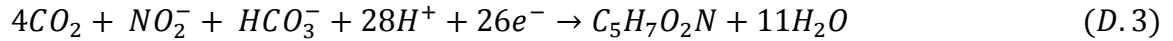
as the sole source of carbon, nitrogen, and energy under aerobic conditions, while predicting the stoichiometric equation that describes the system. The electron donor, electron acceptor, and protein synthesis equations can be represented by



$$\Delta G^\circ = 98.08 \text{ kJ/mol}$$



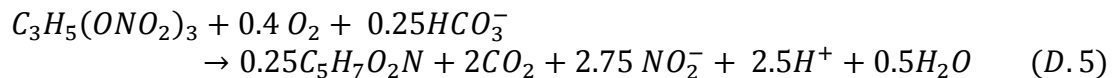
$$\Delta G^\circ = -78.32 \text{ kJ/mol}$$



The coefficient A can be calculated based on the formula

$$A = -\frac{\frac{\Delta G_p}{\varepsilon n} + \frac{\Delta G_c}{\varepsilon}}{\varepsilon \Delta G_{rex}} \quad (D.4)$$

Where  $\Delta G^\circ_{rex} = -78.72 - 98.08 = -176.4 \text{ kJ/eq}$ ,  $\Delta G^\circ_p = \Delta G^\circ_{\text{pyruvate}} - \Delta G^\circ_{ed} = 35.09 - 98.08 = -62.99 \text{ kJ/eq}$ ,  $\Delta G^\circ_c = 14.5 \text{ kJ/eq}$ .  $N = -1$  since  $\Delta G^\circ_p < 0$ .  $\varepsilon$  represents the energy transfer yield for the reaction and it is very commonly assumed to be 0.6 (69). However, recent research (52) indicates that for compounds following widespread catabolic pathways, the best fit value for energy transfer efficiency was 0.37, and not 0.6. Based on this new finding, A can be calculated as 0.24 and  $f_s^0$  can be calculated as  $f_s^0 = 1/(1+A)$ . Then,  $f_s^0$  is 0.80. Based on this parameter, the complete stoichiometric equation can be written as



The cell yield can be calculated as 0.25 mmol cells/mmol NG, or 28.25 mg cells per mmol of NG.

To experimentally verify this result, a mixed culture capable of growing on NG as the sole source of carbon and nitrogen was isolated from a contaminated soil sample from Kenvil, NJ. The following section describes the experimental procedures and the results obtained with this mixed culture.

## D-2 METHODOLOGY

---

*Chemicals.* NG, 1,2 DNG, and 1,3 DNG standard solutions in acetonitrile were purchased from Cerilliant (Round Rock, TX). Methanol Chromasolv plus for HPLC > 99.9% (Sigma Aldrich, Montana) was used for chromatography; glycerol used for NG manufacturing was spectrophotometric grade > 99.5% and was purchased from Acros Organics.

*Nitroglycerin Synthesis.* NG was synthesized using sulfuric acid, nitric acid, and glycerol with a proportion of 3.3 to 2.7 to 0.3 (V/V/V), respectively (86). Components were mixed in a constant temperature chamber at 25°C for 60 minutes and the resulting NG NAPL was then transferred to filtered DI water using a pipette. This process was repeated until the pH was stable and low nitrate concentration was detected in the aqueous phase. Following, the NG was pipetted into a sterilized water bottle and was allowed to dissolve in a shaker for at least three days, to produce a dissolved NG stock solution.

*Analytical.* Nitroglycerin, 1,2DNG, 1,3DNG, 1 MNG, and 2 MNG were measured using Agilent HPLC equipped with a Supelguard guard column (10 x 4.6 mm, Supelco,



Bellefonte, PA), a Supelco LC-18 column (250 x 4.6 mm, 5  $\mu$ m, Supelco) and a UV detector. Methanol-water (50% v/v) was used as the mobile phase with a flow rate of 1ml/min and the wavelength was set at 214 nm. Nitrite and nitrate were analyzed using a Dionex IC equipped with a IonPac AS14A Anion-Exchange Column (Dionex, CA) at a flowrate of 1ml/min. Total protein was quantified using the Micro BCA Protein Assay Kit (Pierce Biotechnology, IL).

*Culture Media.* Mineral medium consisted of (in grams per liter)  $\text{Na}_2\text{HPO}_4$ , 2.66;  $\text{KH}_2\text{PO}_4$ , 1;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.02;  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.01;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.003; and 1.0 ml of trace elements solution per liter. The trace elements solution contained the following (per liter)(83): 0.1 g of  $\text{HBO}_3$  and 0.05 g (each) of  $\text{CaSO}_4 \cdot 5\text{H}_2\text{O}$ ,  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , and  $\text{Na}_2\text{MoO}_4 \cdot 6\text{H}_2\text{O}$ . Mixed bacterial culture was enriched from contaminated soil from Kenvil, NJ, with NG as the sole source of carbon, nitrogen and energy. After several months of enrichment, a shake flask experiment with NG (0.35 mM) in mineral medium (in duplicate) was conducted to develop a mass balance on total nitrogen during biodegradation. Here, molar amounts of nitrogen contained in NG, DNG, MNG, nitrite, and nitrate were measured at times 24h, 264h, and 312 h.

### D-3 RESULTS AND DISCUSSION

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The objective of developing a nitrogen mass balance was to investigate the stoichiometry of NG biodegradation when used as a sole source of carbon and nitrogen. DNG concentrations were measured as 0.03mM at time 24h, and 0.056mM at time 312h. The MNG concentrations were measured as 0.003 mM at time 24 h and 0.016 at time 312 h. Protein final concentration was 0.014mM. Since all nitrogen was accounted for at all of the investigated points in time (Figure D-1) results suggest that the only nitrogen-

containing intermediate compounds are the same as those found in cases when additional sources of carbon were added (1,3-DNG, 1,2-DNG, 1-MNG, 2-MNG) (25, 51, 89). This observation is of significance because to our knowledge there are no previous publications that conducted a mass balance to investigate if the proposed degradation pathway for cases in which additional sources of carbon were added and nitroglycerin was only used by bacteria as a source of nitrogen, is also the degradation pathway in cases when bacteria need to use NG as a source of carbon.

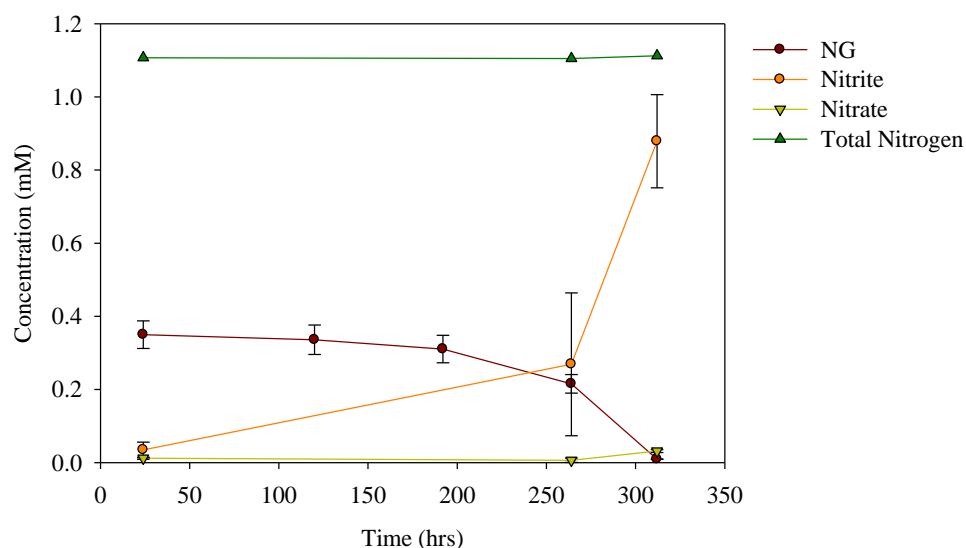


Figure D-1. Nitrogen mass balance during NG biodegradation by mixed culture. Data represent average of replicates  $\pm$  range

Most of NG degradation resulted in nitrite formation and nitrate represented less than 3% of the total nitrogen at 312h, indicating that nitrite can be used as an indicator of NG biological degradation (Table D-1). The presence of nitrate can be probably attributed to the hydrolytic degradation of MNG, which based on previous experiments, will take place at pH values below 7 at room temperature. Nevertheless, other processes

such as nitrification could alter these results and further studies should be conducted to investigate this phenomenon. No nitrification was observed in jar tests containing the isolated microorganisms under aerobic conditions, where nitrite was used as the electron donor indicating that nitrification was not the source of nitrate in this investigation.

**Table D-1. Nitrogen mass balance for mixed culture isolated from contaminated soil (mass %)**

Time (hr)	NG	DNG	MNG	Protein	Nitrite	Nitrate	Total
24	95	1	0	0	3	1	100
264	58	14	1	2	24	1	100
312	3	10	2	3	79	3	100

Data represent average from duplicates

Although over 97% of NG was removed after 312 hours, exponential growth was still being observed in both cultures (Figure D-2). This observation indicates that MNG and DNG that were still remaining after 312 hours were being used by bacteria as substrate and it is expected that if more time would have been given to the experiment, complete mineralization of both MNG and DNG would have been achieved.

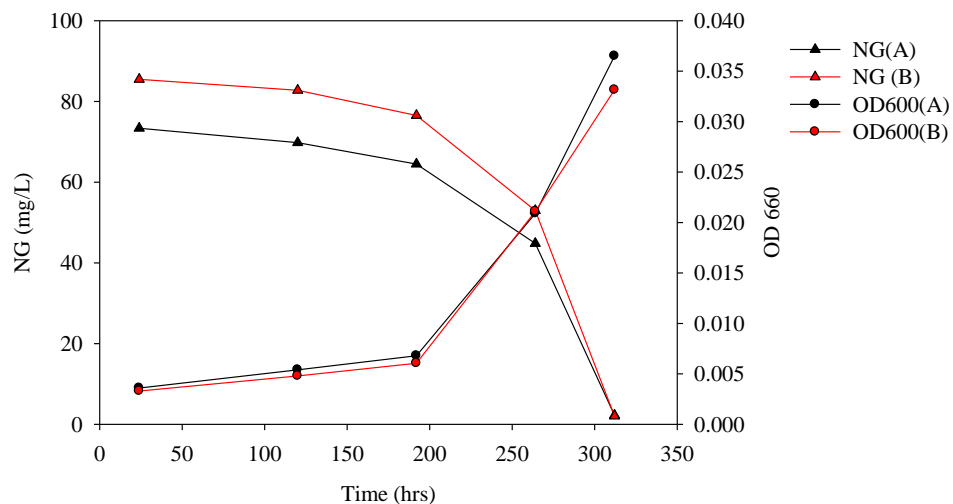
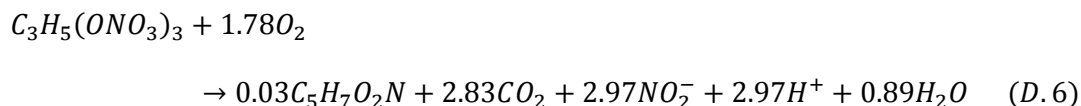


Figure D-2. NG concentration and growth during NG biodegradation

Based on results from this experiment, an approximated stoichiometric equation for the system was derived and it is presented in Equation D.6. Further experiments using a pure culture isolated from this mixed culture are described in Chapter 3.



The cell yield of this reaction is much lower than predicted (only 3.73 mg cells per mmol of NG). Additionally, this reaction requires more oxygen, and releases more nitrite per mol of NG consumed. The difference between observed and predicted result can be caused by the high amount of energy required for the initiation of the degradation mechanism which requires NAD(P)H (52). This result indicates that most of the nitroglycerin molecule is being used for energy resulting in a very low cellular yield and therefore a high production of nitrite, which is also a toxic compound. This finding suggests that nitrite production during nitroglycerin biodegradation *in situ* should be monitored to avoid hazards due to nitrite exposure.

At least three different types of colonies were identified on TSA plates (Figure D-3). During the first few weeks of enrichment, degradation by the mixed culture was slow and the lag phase was significant (about 300 hours) (Figure D-4). When NG concentrations greater than 1 mM were used, no growth and no NG degradation were observed. After eight months of enrichment, however, degradation became much faster, and concentrations even as high as 1.4 mM were degraded successfully. Results indicate that NG can be inhibitory to bacteria at concentrations that are well below the solubility of the compound. This phenomenon would make biological degradation very difficult near the NG source.

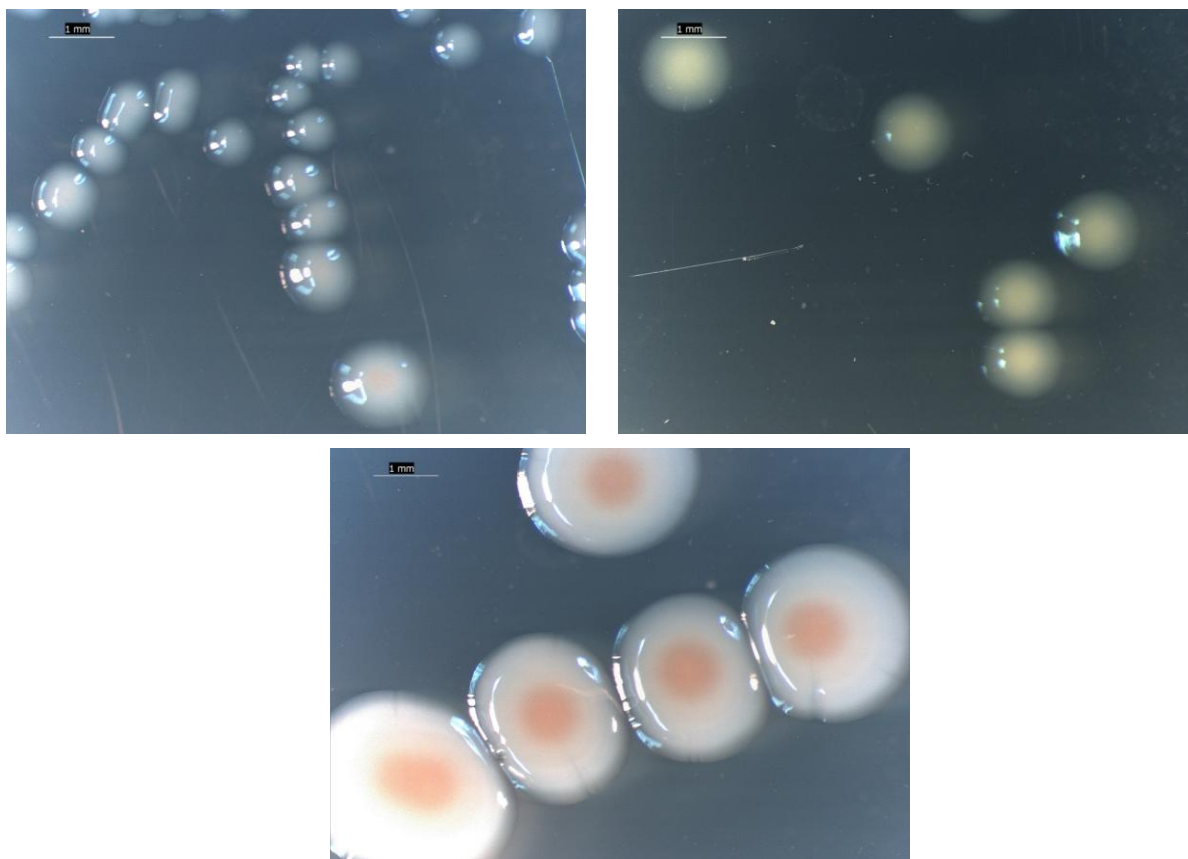


Figure D-3. TSA plates from NG enrichment prior to isolation of *Arthrobacter* JBH1

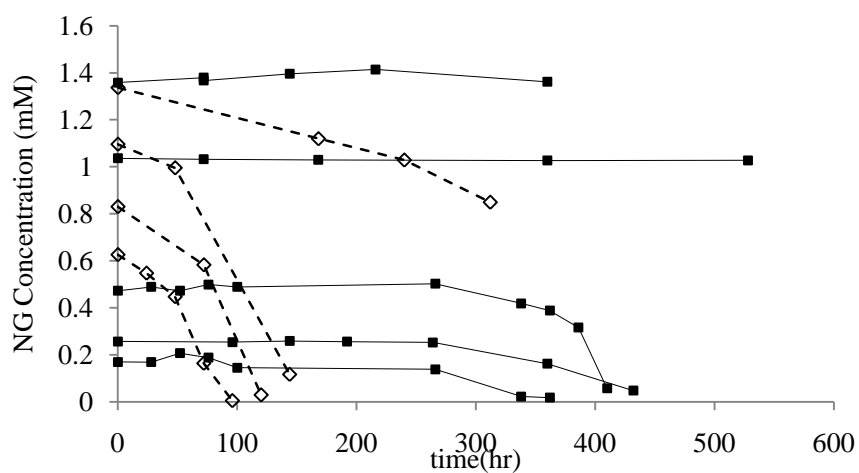


Figure D-4. Early microbial enrichment capable of growing on NG (solid lines) at different initial NG concentrations and microbial enrichment after 8 months (dashed lines)

Results presented in this section show that microbes isolated from contaminated soil can successfully grow on NG as the sole source of carbon, nitrogen and energy. However, to fully understand the mechanism used by microbes to grow on NG, it is necessary to isolate a pure culture, determine the biodegradation pathway and the enzymes involved. The isolation of an NG degrading culture from this enrichment is described in Chapter 3.

## APPENDIX E: PH AND SUBSTRATE INHIBITION

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Biologic NG degradation is expected to follow Michaelis-Menten enzyme kinetics which accounts for enzyme specificity and substrate saturation. This model is based on the fact that one substrate molecule will react with one enzyme forming an intermediate compound (69)



Where E is the enzyme, S is the substrate and ES is the intermediate compound. The intermediate compound will react to form the product (P) and release the enzyme



The substrate degradation rate is then given by

$$\frac{d[S]}{dt} = -\frac{k_2 E^o [S]}{K_M + [S]} \quad (E.3)$$

Where  $E^o$  is the total enzyme concentration initially present and  $K_M$  is the Michaelis-Menten Coefficient which is (69)

$$K_M = \frac{k_{-1} + k_2}{k_1} \quad (E.4)$$

A similar model was later proposed for biological degradation based on enzyme kinetics, which also accounts for substrate saturation. The Monod kinetic model can be used to describe the substrate degradation rate

$$R = \frac{d[S]}{dt} = -\frac{kX[S]}{K_s + [S]} \quad (E.5)$$

where  $k$  is the maximum substrate utilization rate,  $X$  is the biomass concentration and  $K_s$  is the half-velocity concentration at one half the maximum substrate utilization rate (84).

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## E-1 METHODOLOGY

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JBH1 was grown on LB medium at room temperature to a cell density (OD<sub>600</sub>) ~10. Cells were washed three times with phosphate buffer (pH 7.2). Following, cell extracts were obtained using the french press and were centrifuged at 26000 x g for 60 minutes. Supernatant (1.6 mg protein /mL) was kept on ice at all times. NADPH solution (10 mM) was prepared just prior to initiating the experiments. 2 mL centrifuge tubes containing mineral medium, NG stock solution (different amounts to reach NG initial concentrations of 21, 27, 58, 79, 146, and 185  $\mu$ M) and 80  $\mu$ L of cell extracts, were amended with 20  $\mu$ L NADPH solution (10 mM) to a final concentration of 200  $\mu$ M (total sample volume of 1 mL). Immediately, 100 $\mu$ L of sample were collected and placed in a separate vial containing 350  $\mu$ L of UP water and 50 $\mu$ L of HCl (1N) to quench the reaction. After 5 minutes, a second 100  $\mu$ L aliquot was taken from each centrifuge tube and again was placed in a vial containing acid. All samples were kept at -20°C until analyzed using HPLC. Separately, to test for NG inhibition at higher NG concentrations serum vials containing 20mL of mineral medium were inoculated JBH1 at pH 7.2. Experiments to investigate the effect of NG concentration on its biodegradation by this strain were conducted by varying the NG concentration (0.26, 0.33, 0.82, 1.04, and 1.22mM) and keeping all other parameters (initial biomass concentration, pH, temperature, and oxygen



concentration) the same. The effect of pH on NG biodegradation by *Arthrobacter* sp. strain JBH1 was studied using serum vials containing 20 mL of mineral medium. 1 mL of NG solution was added to each vial and it was later inoculated with JBH1. pH in the vials was adjusted using HCL 1N. The pH values investigated were 5.1, 5.7, 6.2, 6.5 and 7.2. No higher pH was investigated to avoid hydrolytic degradation. An abiotic control was used at a pH of 7.2.

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## E-2 RESULTS AND DISCUSSION.

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Cell extracts were used to determine the kinetic parameters that describe the denitration of NG by JBH1. Here, low concentrations of NG were used to produce a Lineweaver-Burk plot. The Lineweaver-Burk plot uses the relationship presented in equation E.5, and transforms it further to

$$\frac{X}{R} = \frac{K_S}{k} \left( \frac{1}{[S]} \right) + \frac{1}{k} \quad (\text{E.6})$$

Here, a plot of  $1/[S]$  vs  $X/R$  is used to determine the kinetic parameters  $k$  and  $K_S$  (Figure E-1). NADPH was used instead of NADH based on previous observations of NG degradation in JBH1 cell extracts in the presence of NADPH and NADH, which indicated denitration enzymatic processes involved in NG denitration prefer NADPH to NADH.

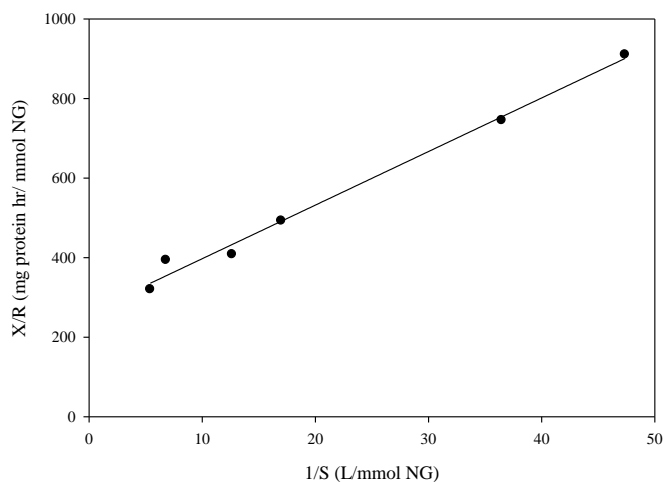


Figure E-1. Lineweaver-Burk plot of NG transformation by cell extracts from JBH1 and NADPH (200  $\mu$ M)

Sigma Plot was used to analyze the curve based on a linear equation of the form  $f = y_0 + ax$ . The  $R^2$  is 0.9904. Coefficients calculated were  $y_0 = 262.6$  with a standard error of 17.3 ( $P = 0.0001$ ,  $t = 15.1673$ ) and  $a = 13.5$  with a standard error of 0.67 ( $t = 20.3355$ ,  $P < 0.0001$ ). Based on these coefficients,  $K_s$  was calculated as 0.051 mM and  $k$  is 0.0038 mmol NG/ mg protein hr.

Experiments conducted at higher NG concentrations using whole cells indicated that NG denitration rates were slower than expected (based on the previous analysis) at very high NG concentrations ( $> 1$  mM). This observation is consistent with substrate inhibition, in which the substrate concentration is so high that it binds to the enzyme on two different sites. This mechanism can be modeled using Andrews kinetics as follows (9)



After algebraic manipulation of these equations, the substrate degradation rate is then

$$\frac{d[S]}{dt} = \frac{k_2 E^o [S]}{K_M + [S] \left(1 + \frac{[S]}{K_2}\right)} \quad (E. 10)$$

Where  $K_2$  is the dissociation constant for the inhibitory SES complex (22). Similarly, the rate of biological substrate consumption can be described by

$$R = \frac{d[S]}{dt} = \frac{kX[S]}{K_S + [S] \left(1 + \frac{[S]}{K_I}\right)} \quad (E. 11)$$

Where  $K_I$  is the inhibitory concentration of the inhibitory substrate. This model is also called the Haldane-Andrews model and has been previously used to model the growth kinetics of *Pseudomonas* spp. and of *Gliomastix indicus* MTCC 3869 for benzene degradation (46) and for p-cresol degradation (76), respectively, which are both inhibitory at high concentrations. There are other inhibition models that can be used to describe substrate inhibition (Webb, Yano, Aiba and Teisser type)(76). Yano uses the model proposed by Haldane but generalizes it further by including the formation of additional inactive complexes,  $ES_3$ ,  $ES_4$ , and so on (27). Web goes a little further by predicting that  $ES_2$  will still react to form  $ES+P$  (27). Both models require the identification of an additional constant ( $K_s$ ,  $K_I$ ,  $k$ , and  $K$ ) and in general were found to have less well-fit to experimental data (27). The Aiba model, on the other hand, is an empirical approximation (obtained from experimental observations) and cannot be explained by enzymatic kinetics (3).

Equation E.11 was further rearranged to

$$\frac{K_s}{k} + \frac{[S]}{k} + \frac{[S]^2}{kK_I} = \frac{[S]x}{R} \quad (E.12)$$

Using the initial measured degradation rate, the initial biomass concentration, and the initial substrate concentration, the quadratic solution for the plot of  $[S]x/R$  versus  $[S]$  can be used to identify the coefficients  $k$ ,  $K_s$ , and  $K_I$  (Figure E-2) (45)

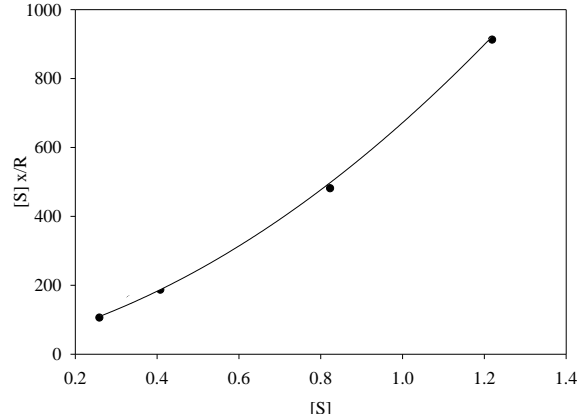


Figure E-2. Experimental results for initial biodegradation rates associated to initial substrate concentrations fitted to Andrew's model

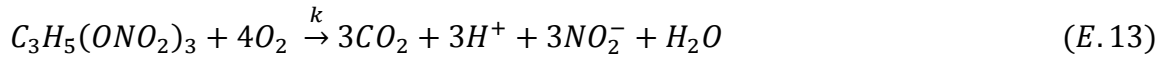
The statistical package included in SigmaPlot was used to determine the parameters of the curve of the form  $f=y_0+ax+bx^2$ , with  $y_0=-16.02$ ,  $a=-257.68$ , and  $b=-398.1150$  and  $R^2$  of 0.9977 (standard error 20.99). Thus, the kinetic coefficients were calculated as  $a=1/k$  ( $k=0.0038$  mmol/mg protein hr);  $y_0=K_s/k$  ( $K_s=0.062$  mM); and  $b=1/(K_I k)$  ( $K_I=0.64$  mM).  $K_s$  and  $k$  are consisted with those predicted earlier with cell extracts at lower NG concentrations. The effect of substrate inhibition resulted in a decrease in initial observed degradation rates from 0.0024 mmol/mg protein hr at an initial NG concentration of 0.335mM to 0.0013 mmol/mg protein hr at an initial NG concentration of 1.22mM.

The comparison of the experimental results with modeled predictions using equation E.11 (with substituted parameters) is presented in Figure E-3. NG inhibition on biodegradation was previously observed by Accashian et al.,(2) for a mixed culture.

However, these authors reported NG toxicity at concentrations above 0.3 mM and this microorganism did only show signs of toxicity at concentrations higher than 0.5 mM.

Results from experiments conducted at different pH values indicated that NG could be effectively biodegraded by this pure culture at all pH values investigated and that degradation rate increases as a function of pH, obtaining a maximum degradation rate at pH 7.2. This observation indicated that pH affects the kinetics of the system.

Based on the NG oxidation equation



it is hypothesized that both oxygen and pH will affect the rate of reaction. Because all experiments were conducted under fully aerobic conditions (open to atmosphere and completely mixed), the effect of oxygen on degradation rate cannot be determined from this set of experimental data. The effect of pH could be then modeled as follows

$$\frac{d[S]}{dt} = \frac{kX[S]}{K_S + [S] \left(1 + \frac{[S]}{K_I}\right)} \cdot [H^+]^a \quad (E.14)$$

If all other parameters (except pH) are the same, an approximation can be done using initial conditions. Then,

$$A = \frac{kX[S_o]}{K_S + [S_o] \left(1 + \frac{[S_o]}{K_I}\right)} \quad (E.15)$$

$$\frac{d[S_o]}{dt} = A \cdot [H^+]^a \quad (E.16)$$

And

$$\log(\text{Rate}_o) = \log A + a \cdot \log[H^+] \quad (\text{E.17})$$

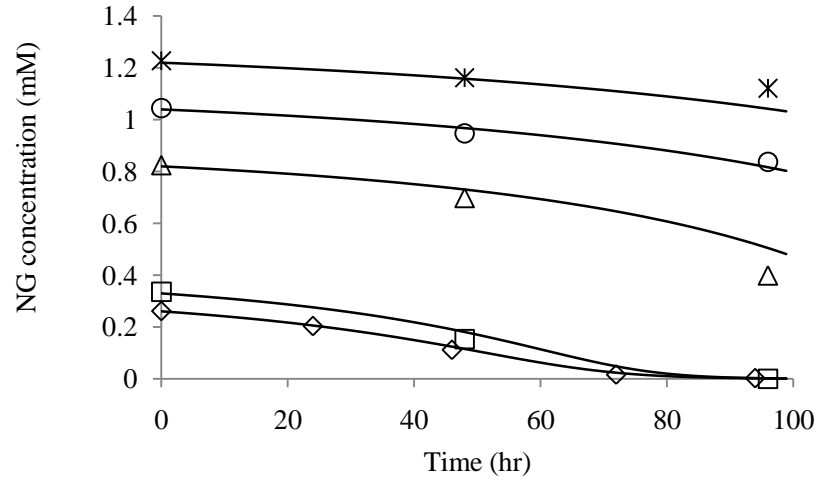


Figure E-3. Modeled NG biodegradation results using the predicted  $k$ ,  $K_M$ , and  $K_I$  values (lines) compared with experimental results (markers)

Based on this approximation, it was determined that  $a = -0.1535$ . It is negative indicating higher  $[H^+]$  will hurt the reaction, which means higher pH is more favorable (Figure E-4).

This result indicated that maximum NG degradation rate is affected by pH.

Previously it was found that  $k$  was 0.0038 mmol/mg biomass hr, at a pH of 7.2.

This was only an observed value; now using the obtained value  $a$ , we obtain the new  $k$ , as

$$k_{obs} = 0.0038 = k \cdot [H^+]^{-0.1535} \quad (\text{E.18})$$

And  $k = 0.0003$ .

The overall equation that represents the degradation of NG by *Arthrobacter* sp. Strain JBH1 coupled to bacterial growth is then

$$\frac{d[NG]}{dt} = \frac{0.0003X[NG]}{0.062 + [NG] \left(1 + \frac{[NG]}{0.64}\right)} \cdot [H^+]^{-0.1535} \quad (\text{E.19})$$

The growth equation is then

$$\frac{d[X]}{dt} = \frac{Y \cdot 0.0003X[NG]}{0.062 + [NG] \left(1 + \frac{[NG]}{0.64}\right)} \cdot [H^+]^{-0.1535} - bX \quad (E.20)$$

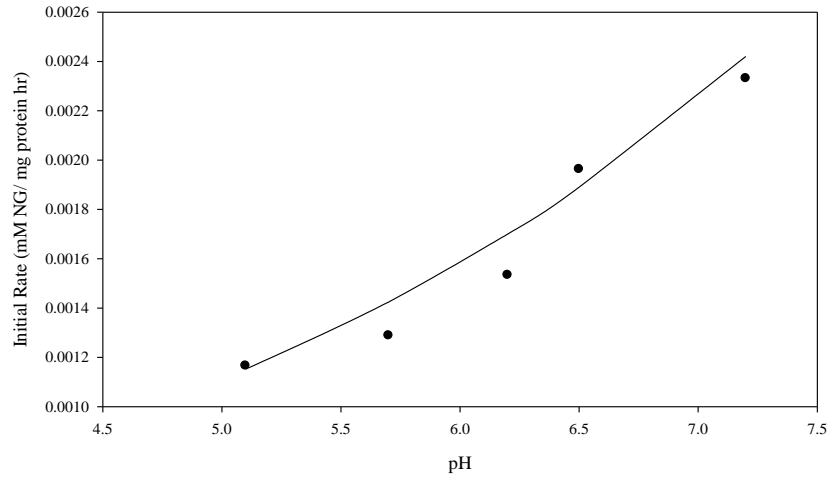


Figure E-4. Initial NG degradation rate as a function of pH for *Arthrobacter* JBH1. Comparison of experimental (markers) v. modeled results (solid line)

With  $b=0.004/\text{hr}$  and  $Y=13\text{mg biomass/mmol NG}$ . Matlab® was used for modeling purposes using the function ode45, which utilizes a 4<sup>th</sup> order accuracy Runge-Kutta method. The concentrations of carbon and nitrite were obtained through mass balances on nitrogen and carbon (Figure E-5).

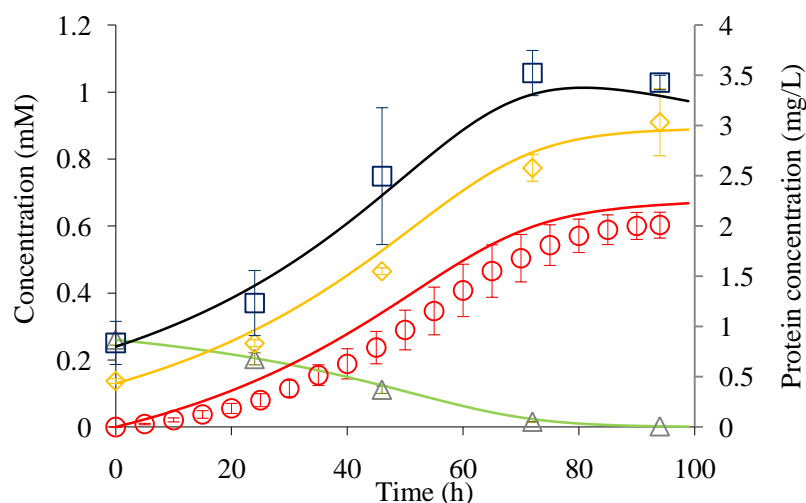


Figure E-5. Comparison of experimental (markers) versus modeled results (solid line) using substrate inhibition models and including pH effect on degradation. Green: NG concentration, mM. Blue: biomass concentration (mg/L). Yellow: Nitrite concentration (mM). Red: Carbon dioxide (mM)

Substrate inhibition was evident based on the experimental results obtained. The enzymatic model in which an enzyme is bound by the substrate at two sites in the presence of high amounts of substrate represents the experimental data very well. Here, the maximum degradation rate decreases as a function of NG concentration, with an inhibitory concentration around 0.64 mM. This result is of great significance because it means that NG solubility concentration (~6.6 mM) is inhibitory for this microorganism, resulting in a very low degradation rate near contaminant plumes. The maximum growth rate obtained was similar to that obtained for a mixed culture by Accashian (2) using similar conditions (pH=7, similar initial concentration and aerobic conditions).

The effect of pH on NG biodegradation by *Arthrobacter* sp. Strain JBH1 was studied. As expected based on the NG oxidation equation,  $[H^+]$  has a negative effect on the degradation rate (negative exponent). This observation indicates that the degradation rate



is more favorable at higher pH values. However, pH values of 7.5 and above were not investigated here because there is evidence of NG hydrolytic degradation at room temperature and pH values higher than 7.5 in less than 48 hours and it was only desired to study the biological degradation of this compound. This model is only valid for the pH range investigated (5.1 to 7.2) and more experimental data are required to produce a model that covers a larger pH range.

The model obtained through this investigation and used to predict biodegradation rate/biomass production had a very good fit with experimental data obtained. However, the calculated concentrations of CO<sub>2</sub> and nitrite were overestimated during the intermediate time steps and were only close to experimental results at the end of the degradation process. This result can be explained by the fact that the mass balances did not take into account the formation of the intermediate compounds (DNG and MNG). Although the concentrations of these intermediates are low as compared to the initial NG concentration, some carbon and nitrogen is in the form of these compounds and therefore not all N or C can be accounted for. However, there are not enough experimental data that can be used to estimate the degradation rates of the intermediate compounds. Therefore, this approximation is used to represent degradation kinetics by this strain, being this one of the limitations of the proposed model. Other limitations of the model are due to the lack of experimental data to relate rate with oxygen concentration and temperature (all experiments were conducted at temperatures between 21 and 22<sup>0</sup>C and exposed to ambient air conditions). It is expected then that a new degradation rate can be found that is a function of oxygen saturation (assuming it also follows a Michaelis-Menten Mechanism)

$$\frac{d[NG]}{dt} = \frac{0.0003X[NG]}{0.062+[NG]\left(1+\frac{[NG]}{0.64}\right)} \cdot [H^+]^{-0.1535} \cdot \frac{[PO_2]}{K_o+[PO_2]} \quad (E.21)$$

And  $K_o$  could be obtained from experimental data. The effect of temperature on  $k$  should also be investigated in the future. This investigation is of great importance for the understanding of NG degradation as a sole source of carbon and nitrogen, because it investigates the effects of pH and NG concentration on biodegradation, which as observed here, are key factors on the degradation rate.

The ability of *Arthrobacter* sp. Strain JBH1 to grow on NG as a sole source of carbon and nitrogen, to tolerate relatively low pH and high NG concentration presents a foundation for the study of potential remediation strategies that involve biodegradation and natural attenuation at NG contaminated sites. However, many questions regarding the degradation of NG in porous media still remain and it is unknown if *in-situ* biodegradation rates are significant enough for NG bioremediation and natural attenuation to be considered as remediation alternatives at contaminated sites. Additionally, the precise denitration mechanism used by JBH to grow on NG is still unknown, and understanding of this mechanism could answer many questions regarding the recalcitrance of NG intermediates *in situ* and the use of NG as a growth substrate instead of cometabolic substrate. These questions will be addressed in the following chapters.

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